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FORM PTO-1390 (REV. 12-2001) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 17642-59

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 10/089534

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/IT00/00382	28.September.2000 (28.09.2000)	01.October.1999 (01.10.1999)

TITLE OF INVENTION
BIO-ARTIFICIAL SUBSTRATE FOR THE PRODUCTION OF ANIMAL AND, IN PARTICULAR, HUMAN TISSUES AND ORGANS

APPLICANT(S) FOR DO/EO/US
ARMATO, Ubaldo; MIGLIARESI, Claudio; MOTTA, Antonella; and

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [x] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [x] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [x] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [x] is attached hereto (required only if not communicated by the International Bureau).
   b. [x] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [x] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [x] Other items or information:
    International Application with Annexes to IPER incorporated; extra set of drawings;and International Preliminary Examination Report

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
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21. ☒ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	- 20 =		x **$18.00**	$	
Independent claims	- 3 =		x **$84.00**	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$	
TOTAL OF ABOVE CALCULATIONS =				$ 890.00	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.			+	$ 445.00	
SUBTOTAL =				$ 445.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$ 445.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$ 445.00	
			Amount to be refunded:	$	
			charged:	$	

a. ☒ A check in the amount of $ 445.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 16-2230 in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-2230 . A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

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45,267
REGISTRATION NUMBER

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21/PRTS 1

BIO-ARTIFICIAL SUBSTRATE FOR THE PRODUCTION OF ANIMAL AND, IN PARTICULAR, HUMAN TISSUES AND ORGANS

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TECHNICAL FIELD

This invention, concerning the biomedical sector, refers in particular to a substrate for cell and tissue culture which favours the survival, proliferation, differentiation and correct functioning of cells of specialised tissues.

This substrate is ideally employed in the medical and surgical fields since it can be used for the regeneration of organs and tissues to be transplanted in humans or for the setting up of bio-artificial organs to be functionally connected with the organism.

One particularly advantageous application of this invention is the production of a regenerating artificial skin to be grafted onto patients with major burns or suffering from diseases that involve the degeneration of the cutaneous tissues.

BACKGROUND ART

The skin is the most conspicuous organ of the human body, accounting for 16% of its mass and having the largest surface area, and it grows and renews itself at a constant rate. A complete renewal cycle of the superficial layer of cutaneous cells (epidermis) does in fact take place every three-four weeks.

Contrary to what may appear from a cursory analysis of its characteristics, the skin is also one of the most complex organs of the human body since it consists of extremely specialised cell types that perform a series of very different, specific, and well-balanced functions ensuring the maintenance of the body's outer covering, protection against infection, heat and ultraviolet radiation, regulation of body temperature, water loss (sweating), secretion of fats (sebum) and production of vitamin D.

The outer layer of the human skin, which is known as the epidermis, contains no blood vessels and consists mainly of closely aggregated cells called keratinocytes. Other cells present in lesser quantities in the epidermis are melanocytes, Merkel cells and Langerhans cells. The so-called "basal lamina", consisting of type IV and VII collagen, fibronectin, etc., marks the boundary with the underlying dermis.

From a structural point of view, the epidermis consists of five cellular layers

From a structural point of view, the epidermis consists of five cellular layers which from the surface downwards are the *stratum corneum*, the *stratum lucidum*, the *stratum granulosum*, the *stratum spinosum* and the *stratum basale* or *germinativum*, and it is thanks to these layers that the skin can perform its role of mechanical protection of the human body. The natural history of any keratinocyte in the epidermis involves a progressive differentiation as it moves upwards from the *stratum germinativum* to the *stratum corneum*, passing through all the intermediate layers in sequence and finally being transformed into a dead corneocyte and being sloughed off by friction or during washing. The *stratum germinativum* is in direct contact with the basal lamina and is the site where new cells are generated. As already mentioned, the basal lamina represents the boundary between the epidermis and the subjacent layer of connective tissue known as the dermis or *corium*.

This layer is extremely well vascularised and also contains receptors connected with nerve fibres that allow the sensorial reception of mechanical, physical and chemical stimuli by the skin. The mechanical receptors include the Pacinian corpuscles and Meissner's corpuscles, the latter being particularly responsible for tactile sensitivity. Blood vessels and nerves are particularly abundant in the outermost layer of the dermis which is adjacent to or in contact with the basal lamina.

The dermis too contains cells, including fibroblasts, which are scattered within a matrix consisting of various fibrous proteins, such as collagen and elastin, and of components with an amorphous ground substance, such as glycosaminoglycans (GAG) and proteoglycans.

Fibroblasts perform very important roles since, as well as producing the precursors of the collagen and elastic fibres and the components of the amorphous matrix, they also secrete growth factors and cytokines that are capable of affecting the functioning of the keratinocytes and endothelial cells of the blood vessels.

The high level of structural complexity of the human skin is undoubtedly the greatest problem to be solved when attempting to produce artificial tissues that reproduce the skin's morphofunctional characteristics. These tissues, commonly known as "artificial skin-grafts" by those working in the sector, should be particularly useful,

and are thus studied very intensely, in relieving the suffering of patients with major burns or those affected by diseases involving significant dermatological implications. The severe scarring and psychological damage caused by the sudden devastation of the body image as a result of major burns or to a lesser degree by diseases with significant dermatological implications, as well as the other functional consequences of the damage to an organ as important as the skin, have led researchers throughout the world to strive for alternative solutions that would favour the healing processes and at the same time slow down the development of disfiguring scars and considerably improve the body image and psychological condition of the patients mostly thanks to the use of skin-grafts in the injured areas, which practically eliminate the risks of rejection.

These risks decrease the more the "artificial skin" resembles the natural skin and when, wherever possible, the patient's own cells, or human cells of other origin, are used. Before arriving at the production of artificial cutaneous tissues, a great deal of work had to be carried out in modelling the natural skin in order to reconstruct the synergisms that take place both in the normal functioning of the organ and in extreme conditions, such as immediately after the occurrence of a serious injury and during the healing process.

The reconstruction of cutaneous skin tissues is not a single process but a combination of a series of dynamic interactive processes that involve epidermal and dermal cells, subcutaneous tissue, and blood vessels. These cells actively communicate with each other by releasing soluble messengers – cytokines – which interact with specific membrane receptors.

The most general classification of a tissue reconstruction process such as that of the skin is made by identifying three distinct phases (Principles of Tissue Engineering, Lanza, R. P., Langer, R., Chick, W. L. 1997, Academic Press, R.G. Landes Company, Austin, Texas, U.S.A.) of reaction of the tissue to the injury: (1) inflammation, (2) tissue formation and (3) tissue remodelling.

The injured tissue is studded with severed and bleeding blood vessels. It is also discontinuous in proportion to the severity of the injury. The first reaction that occurs is the formation of a blood clot. The clot is rich in fibrin which blocks blood loss, evens

out the surface of the injured tissue, and prevents the entry of harmful environmental agents. The platelets in the clot produce mediators, including important growth factors. The fibrin also acts as an aggregating agent for further platelets. Numerous macrophages and neutrophil leukocytes arrive at the site of the injury; their action is important since they clear the injured area of foreign bodies and agents that may further harm the damaged tissues (e.g. bacteria). The neutrophils die spontaneously or due to the effect of toxic factors and are in turn phagocytized by the macrophages that arrive at the site somewhat later (see below).

The initial scenario described above also includes enzymes and oxidants that speed up the various reactions triggered by the interactions between the various parts in play, and act as a buffer where the tissue is most vulnerable, i.e. where foreign bacteria endanger the protective processes set into motion on the raw surfaces by the platelets and neutrophils.

Together with the infiltration of the neutrophils there is also an accumulation of monocyte-derived macrophages, attracted by the local release of particular chemokines. Via the mediating actions of integrin receptors, implemented *ad hoc* by the endothelial cells of the blood vessels, these leukocytes are "caught" locally and induced to cross the blood vessels, to penetrate the tissue matrix, and metamorphose into inflammatory macrophages in order to phagocytize cellular debris and residues of collagen and elastic fibres, the components of the extracellular matrix, the degenerated neutrophils and any pathogens.

Re-epithelization of the wound starts just a few hours after the injury. The epithelial cells that have survived the injury undamaged migrate from their initial site to the surface areas covered by the blood clot, giving rise to a new epithelial layer under which further growth of the connective tissue is possible. To this aim, the epidermal cells undergo structural changes allowing their lateral mobilization by the preliminary dissolution of the desmosomes. At the same time, the epidermal cells surrounding the wound develop a motor system that allows their migratory activities. In the wound areas that remain temporarily uncovered, the injured surface consists of a temporary matrix of fibrin, fibronectin, tenascin, vitronectin and type I collagen.

As the re-epithelization process goes on, the proteins of the basal lamina reappear according to a well-defined sequence, starting from the edges of the wound inwards.

Four days after the injury, so called granulation connective tissue begins to form. This process starts the restoration of the integument as a structure consisting of at least two distinct interdependent layers, an outer epithelium and an inner dermis, the latter being rich in capillaries, macrophages and fibroblasts which cooperate actively with each other. The macrophages are in fact a source of important cytokines which play a role of fundamental importance both in angiogenesis and in the production of new collagen and elastic fibres by the fibroblasts that also regenerate the extracellular matrix that will regulate cellular metabolism. These cytokines, endowed with a high proliferation-inducing power, are commonly considered on the same level as growth factors. The temporary extracellular matrix is rich in substances that further tissue development. Examples of such substances are fibronectin and collagen, together with hyaluronic acid.

At this point of the regenerative process, the fibroblasts in the tissues that surround the wounded area proliferate intensely under the mitogenic stimuli of the cytokines and start migrating towards the injured area, first adhering to and then penetrating into the blood clot through the fibronectin matrix.

A mutual equilibrium is thus established between the fibroblasts and the extracellular matrix, since on one hand the fibroblasts continuously synthesize and enrich the matrix with its fundamental components, their functioning being on the other hand regulated by the matrix constituents.

Chemotactile-like mechanisms regulate the migration of the fibroblasts which produce lamellipods directed towards the source of the stimuli, creating new cellular surfaces and triggering complex mechanisms that result in true cell mobility.

Proliferation of the fibroblasts ceases when the system is sufficiently enriched with both cells and collagen matrix (the latter being by itself responsible for blocking the proliferation of these cells, contrary to the effects of fibrin and fibronectin matrix, which favours fibroblast proliferation).

Tissue regeneration is accompanied by a simultaneous process of neo-vascularisation characterised by the ever increasing branching of proliferating capillaries and the neo-formation of capillary loops.

Angiogenesis is made possible by the local release of specific cytokines which stimulate the proliferation of capillary endothelial cells. Angiogenesis allows the tissue to be permeated by oxygen and by the nutritional substances that are indispensable for its survival and correct functioning. Only recently has it become possible to identify and track the molecules that play a fundamental role in angiogenesis. These include, amongst others, angiopoietin-1 (AP-1), vascular endothelial growth factor (VEGF), and keratinocyte growth factor (KGF).

Another phase in which the concerted reorganisation of the tissues and the interactions between the various types of cells involved are particularly intense are the contraction of the granulation tissue filling the wound and the reorganisation of the matrix, during which the wound fibroblasts take on the role and the features of the smooth muscle cells, thus becoming known as myofibroblasts and giving the granulation tissue the ability to contract.

This takes place at the same time as the compacting of the connective tissue, which is caused by the formation of cross-links that join together adjacent collagen fibers. A collagen fiber network is produced which, thanks to the interactions with the matrix, can thus support the contractile stimuli imparted by the myofibroblasts.

The remodelling and catabolism of the collagen fibers constitutes the driving phenomenon that leads to the formation of the scar. This process, however, occurs rather slowly and its functional result is only partly satisfactory. In fact, after the first three weeks following the injury, when the accumulation of fibrillary collagen takes place at maximum speed, the scar tissue has acquired only thirty percent of the strength of the undamaged skin. When it reaches its completion, the scar tissue achieves a resistance only amounting to 70% as compared with that of undamaged skin.

With a scenario such as the one reported above, which does not describe all the micro-phenomena involved in full detail, the production of artificial human skin using synthetic methods represents an extremely difficult challenge.

The use of human skin from living or cadaver donors for the treatment of skin injuries has been practiced for quite some time, although it involves the rejection on the part of the host, not to mention the considerable risk of infections that may even be lethal (e.g. hepatitis C, AIDS, etc.).

The "dermo-epidermal substitutes", more commonly known as "artificial skin" are the result of meticulously perfected cell culture procedures on polymeric supports or substrates often used for subsequent applications onto injury sites. The spaces left free by the mesh of the fabric are sufficient, once proliferation has begun, to favour the population of the polymeric surface as quickly as possible.

The following relevant dermal substitutes are available on the market: Dermagraft®, Allograft® and Alloderm®.

Alloderm® is a dermal substitute, consisting of human donor dermis from which all living cells of epidermis have been removed, which can be preserved at a low temperature, thawed when required and be used in combination with a thin layer of host epidermal cells. The preparatory procedure also involves the inactivation of any virus that might have been originally present in the tissue taken from the donor (generally a cadaver).

The basic protein structure of the dermis, albeit devoid of cells, is preserved by the preparation process. The material is re-hydrated with a saline solution prior to being grafted onto the burn wound site. Clinical applications are limited. The possibility of infections, due to the human origin of the product, cannot be completely excluded. The production process is extremely complex and the quality level plays a highly critical role.

Dermagraft® and Allograft® present the same problems as those described for Alloderm®.

Integra® is another dermal substitute. It consists of an inner layer, 2 mm thick, made up by a combination of bovine collagen fibres and a GAG, chondroitin-6-sulphate, with 70-200 μm diameter pores, and is biodegradable. Its outer layer consists of a silicone polymer that permits water vapour transfer.

Integra® is intended for applications on deep burns, and the silicone outer

membrane must be replaced after 2-3 weeks with an auto-graft of epidermal cells. Clinical experience is rather limited at present: the occurrence of suppurative processes immediately beneath the silicone membrane has been frequently reported, and the production process is extremely complicated and costly.

In addition, the use of collagenous materials of bovine origin exposes the patient to the risk of spongiform encephalopathy, also known as "mad cow disease".

The document US-A-5,266,480 describes a three-dimensional culture of fibroblasts on a polymeric substrate (stromal matrix) made from one of the following materials: nylon (polyamides), dacron (polyesters, polystyrene, polypropylene, polyacrylates, polyvinyl compounds such as polyvinyl chloride, polycarbonates, teflon, termanox, nitrocellulose, catgut, cotton, cellulose, gelatin and dextrane. With the exception of cotton fibre, catgut, cellulose, gelatin and dextrane, these are all polymers of synthetic origin, processed in such a way as to produce a fibre. Pretreatment of the matrix before cell inoculation is also suggested, together with the enrichment of the cell cultures with proteins of various kinds, such as collagen, glycoproteins, glycosaminoglycans (GAGs) and other similar materials in order to speed up the cell proliferation process. This patent stresses the importance of finding a substrate that acts as a support in such a way as to leave sufficient space to prevent the cells from being trapped and from stopping the production of the growth factors necessary to sustain proliferative processes. Thus, growth factors will not have all to be added to the matrix from the beginning, since the cells themselves will continuously produce them, creating right from the start the steady-states that are proper of the skin. The stromal cells are explanted from organs and separated according to conventional methods. One serious disadvantage of this invention is the proposed use of synthetic and non-re-absorbable substrates, constituting to all extents and purposes foreign bodies right from the time of grafting, with the consequent risk of rejection reactions to these materials. Another disadvantage of this process is that it is extremely complicated and requires several cell inoculation stages in order to obtain a fully formed cutaneous tissue consisting of the necessary amount of different cells that carry out the various roles they would perform in healthy and fully developed tissue. Particularly as far as skin is concerned, this

invention also requires the use of cells explanted from the foreskin of newborns, obtained by means of circumcision operations.

The document US-A-5,902,741 describes three-dimensional cultures of cartilaginous cells set up on a biocompatible matrix of non-living material in the presence of TGF-ß.

The stromal cells cultured according to this patent include chondrocytes, chondrocyte progenitors, fibroblasts, endothelial cells, macrophages, monocytes, bone marrow stromal cells and others.

These cells are induced to proliferate by making them adhere to a rigid structure consisting of a biocompatible polymer until its surface is completely covered. This rigid structure contains spaces that are then filled by the stromal cells. According to this patent, once these stromal cells have completed the formation of a solid tissue covering the biocompatible rigid structure, they begin to secrete growth factors and regulatory factors and are therefore ready to act as a support for the implantation of previously cultured *in vitro* cells, which will thus enjoy the ideal conditions to proliferate and/or differentiate according to the specific requirements of the tissue regions in which they are located.

One disadvantage of this invention in order for it to prove successful is the need to resort to synthetic materials, which do not decompose, dissolve or disappear in any way after implantation.

A further disadvantage of this invention is that, although intended for the reconstruction of an organ or of one or more of its parts, the process by which proliferation and differentiation of specialised tissue cells is achieved is extremely complex since, before the desired cells are implanted, the synthetic material must always be covered with the stromal cells. This represents a transition phase that does not lead directly to the desired result and which takes a fairly long time, meaning that the product is not readily available, which can sometimes be crucial in emergency situations

The patent US-A-4,703,108 describes a process for the preparation of biodegradable matrices in the form of three-dimensional sponges or two-dimensional sheets, for which a type I or II collagen-based material is used, which are freeze-dried

and treated with a reticulation agent such as carbodiimide or a reactive succimidylic ester. After this reticulation reaction, the obtained material is subjected to extreme dehydration conditions after which the spongy or sheet-like material is obtained. This material will also contain a chemical agent chosen from a group that also includes type IV and V collagen, fibronectin and laminin.

An obvious disadvantage of the process briefly described above is that it is extremely complex and quite lengthy.

It also involves two drying stages (the second taking place under extreme conditions) which must be carried out with great care since they can cause irreparable damage to the material, making it useless and forcing the operators to start the procedure all over again, with obvious loss of time and effort.

This last problem makes it difficult to envisage this procedure being carried out at an industrial level or in any case to produce amounts of finished material that exceed those required for laboratory experiments.

A further problem of this procedure is that to obtain the desired result a very wide range of compounds has to be used, some of which are chemical agents that require particular caution.

Another problem in the procedure described in the above-mentioned patent is that collagen is cited as the only material to be used. This material has limitations, however, since its properties and characteristics are fixed thus making it difficult to adapt to different situations.

Yet another problem of this procedure is the need for collagen of bovine origin which can be a vehicle of contagion for spongiform encephalopathy, and may be in any case allergenic. It is also enzymatically demolished, and thus re-absorbed, thereby offering no guarantee that this re-absorption will continue until the collagen has completely disappeared nor that the transplanted and regenerated tissue is restored to its original full functional capability.

The patent US-A-5,670,483 describes a series of stable macroscopic membranes that form by self-assembly due to the alternation of the amphophil peptides, hydrophilic and hydrophobic, found in them. The range of application for these membranes is vast,

including their use as artificial skin.

One disadvantage that these membranes present is that they are not bio-degradable or bio-re-absorbable. Once implanted, their mechanical features remain unaltered and do not correspond exactly to those proper of the human skin; the membranes never achieve a complete integration into the tissue environment in which they are implanted or transplanted.

The document US-A-4,963,489 describes a cell and tissue culture system that can develop three-dimensionally.

The procedure foresees a phase in which cells from a specific tissue are explanted, inoculated and cultured on a three-dimensional stromal matrix. In a first stage, the stromal cells will be grown on the synthetic supporting structure until they reach confluence and completely cover its surfaces, thus providing an ideal scaffold for the growth of the implanted cells. The stroma will contain fibroblasts of foetal origin or from adults; alternatively they will be explanted from a cadaver.

Once again, the stromal cells are cultured on a three-dimensional synthetic matrix which per se is not bio-resorbable. The various options foreseen include the use of cotton and cellulose, which are natural fibres but extremely resistant to the chemical attacks that can be undergone in a natural system such as the human body. The use of gelatine is also anticipated, which is none other than denatured collagen, therefore with the same drawbacks and flaws as those reported for the patent US-A-4703108.

The first shortcoming of the invention described above is the permanence of the synthetic material used as a matrix, even after the culture of the specialised cells has been completed. This can lead to unpleasant consequences for the patient. In the event, for example, of the reconstruction of a portion of a functioning organ of the human body, part of its volume would be always occupied by the inert synthetic material, thus reducing its functional capabilities.

A further drawback of the invention is the fact that it requires complex preparation procedures. Merely to render more biocompatible the synthetic material used as a matrix, the latter has to be covered with a layer of a suitable matter, such as e.g. collagen, which is normally of bovine origin, a time-consuming process that greatly

complicates the pre-transplant work.

For some years now, the scientific and technological world has been studying alternative uses of silk and specifically of one of its proteins, i.e. fibroin, exploiting the particular properties of the re-naturated protein. Its permeability to oxygen, ionic permeability, resistance to proteolytic enzymes and to acid and alkaline solutions, transparency and mechanical properties have been evaluated.

Applications have been proposed in the bio-medical field in the contact lens sector, bandages for burns, artificial corneas and biosensors. Various biocompatibility tests have been performed, albeit fragmentarily: hemocompatibility tests (H. Sakabe et al.: In vivo blood compatibility of regenerated silk fibroin. Sen-I Gakkaishi, vol.45, n. 11, 1989, 487-490), evaluation of the inflammatory potential of fibroin films, as well as the comparison of the fibroin films with two model materials having completely different physico-chemical properties which are polymers-poly(styrene) (PST) and poly-(2-hudroxyethyl methacrylate) (PHEMA) (Santin M et al: In vitro evaluation of the inflammatory potential of the silk fibroin. J. Biomed. Mater. Res., vol. 46, n. 3: 382-389, 1999); cell cultures (Y. Gotoh et al.: Synthesis of poly(ethylene glycol)-silk fibroin conjugates and surface interaction between L-929 cells and the conjugates. Biomaterials, vol.18, n. 3, 1997, 267-271), cell culture and the effect of chemical modification of the arginyl residue in Bombyx mori silk fibroin on the attachment and growth of fibroblast cells and in particular the attachment and growth of mouse fibroblast (L-929) cells on the matrices of the natural silk fibroin and regenerated silk fibroin in which half or almost all of the arginyl residues were modified (Y. Gotoh et al.: Effect of the chemical modification of the arginyl residue in Bombyx mori silk fibroin on the attachment and growth of fibroblast cells. J. Biomed. Mater. Res., vol. 39, n. 3: 351-357, 1998).

Fibroin's resorbability is reported in the document US-A-5,606,019 which describes the use of an elastin and fibroin copolymer as a bio-resorbable material, with a bio-resorption speed varying according to the relative percentages of the two protein polymers in the mixture. In the specifications, the resistance characteristics of the mixture are stressed together with its easy resorbability in the conditions imposed by the

specific region of the human body for which the material should be produced and used.

The range of the applications is extensive and includes fibres, printed objects, membranes to prevent the formation of tissue adherences, bandages for wounds, suture threads and clips.

The first drawback of this invention is that it involves a procedure for the production of the proteins necessary to obtain the bioresorbable material based on genetic engineering techniques, which are complex and include the manipulation of plasmids, their transfer and amplification in prokaryotic cells, i.e. *Escherichia coli*, and phases of refining and separation of the finished product (work-up) that must be carried out with great care. It can be noted that the document US-A-5,606,019, does not suggest the use of fibroin as a substrate for the culture, proliferation and differentiation of cells of any type.

In the document WO-A-98/57676 the biocompatibility, flexibility and resistance to infections of fibroin and of sericin are underlined, and their use for the production of material to cover wounds is described. For such purposes fibroin is used in an non-crystalline form, i.e. with a degree of crystallisation that remains below 10%, or in a powder form.

The patent US-A-5,932,207 describes a transplantation system, suitable for the production of biological parts, particularly organs of living organisms, which basically consists of a scaffold, a component that favours the covering of the scaffold with specialised cells, a set of adhesion materials ensuring a sufficient cellular adherence to the structure and a maturation factor ensuring that the specialised cells that have become part of the new system mature and differentiate when necessary.

Yet again, due to the nature of the adhesion materials, it is necessary to resort to lengthy degradation and resorption processes that are not always successful.

Furthermore, the processes necessary to obtain the transplantation-ready system are complex: it is not easy to envisage how it would be possible to produce such a system in amounts exceeding those needed for laboratory use.

The document WO-A-96/25961 describes the use of collagen fibres for the production of a bio-resorbable extracellular matrix, and document EP-A-0913162

describes a specific use of collagen in the form of fabric, suitable for the repair of tissue defects.

BIBLIOGRAPHIC SOURCES

Akai, H. Ultrastructure of fibroin in the silk gland of larval *Bombyx mori*. Exp. Cell Res., *69*: 219-223, 1971.

Armato, U., Nussdorfer, G. G., Neri, G., Draghi, E., Andreis, P. G., Mazzocchi, G., and Mantero, F. Effects of ACTH and 3',5'-cyclic purine nucleotides on the morphology and metabolism of normal adult human adrenocortical cells in primary tissue culture. Cell Tissue Res., *190* : 187-205, 1978.

Auger, F. A., Lopez Valle, C. A., Guignard, R., Tremblay, N., Noel, B., Goulet, F., and Germain, L. Skin equivalent produced with human collagen. In Vitro Cell Dev. Biol. Anim., *31*: 432-439, 1995.

Berthod, F. and Damour, O. In vitro reconstructed skin models for wound coverage in deep burns. Br. J. Dermatol., *136*: 809-816, 1997.

Burke, J. F., Yannas, I. V., Quinby, W. C. J., Bondoc, C. C., and Jung, W. K. Successful use of a physiologically acceptable artificial skin in the treatment of extensive burn injury. Ann. Surg., *194*: 413-428, 1981.

Canetti, M., Seves, A., Secundo, F., and Vecchio, G. CD and small-angle x-ray scattering of silk fibroin in solution. Biopolymers, *28*: 1613-1624, 1989.

Chevallier, A. and Garel, J. P. Studies on tRNA adaptation, tRNA turnover, precursor tRNA and tRNA gene distribution in *Bombyx mori* by using two-dimensional polyacrylamide gel electrophoresis. Biochimie, *61*: 245-262, 1979.

Clayton, M. C. and Bishop, J. F. Perioperative and postoperative dressing

techniques for Integra Artificial Skin: views from two medical centers. J. Burn. Care Rehabil., *19*: 358-363, 1998.

Coulomb, B., Friteau, L., Baruch, J., Guilbaud, J., Chretien-Marquet, B., Glicenstein, J., Lebreton-Decoster, C., Bell, E., and Dubertret, L. Advantage of the presence of living dermal fibroblasts within *in vitro* reconstructed skin for grafting in humans. Plast. Reconstr. Surg., *101*: 1891-1903, 1998.

Dal Pra, I, Tognana, E., Guerriero, C., and Armato, U. Investigations into mechanisms modulating proliferation, differentiation, and apoptosis in cultured liver, adrenal, skin, and bone cells. Ital. J. Anat. Embryol., *102*: 9-119, 1997.

Eaglstein, W. H. and Falanga, V. Tissue engineering and the development of Apligraf, a human skin equivalent. Clin. Ther., *19*: 894-905, 1997.

Eaglstein, W. H. and Falanga, V. Tissue engineering and the development of Apligraf, a human skin equivalent. Cutis., *62*: 1-8, 1998.

Eaglstein, W. H. Dermagraft treatment of diabetic ulcers. J. Dermatol., *25*: 803-804, 1998.

Fossey, S. A., Nemethy, G., Gibson, K. D., and Scheraga, H. A. Conformational energy studies of beta-sheets of model silk fibroin peptides. I. Sheets of poly(Ala-Gly) chains. Biopolymers, *31*: 1529-1541, 1991.

Fraser, R. D., MacRae, T. P., and Stewart, F. H. Poly-L-alanylglycyl-L-alanylglycyl-L-serylglycine: a model for the crystalline regions of silk fibroin. J. Mol. Biol., *19*: 580-582, 1966.

Iizuka, E. Species specificity of the conformation of silk fibroin in solution.

Biochim. Biophys. Acta, *160*: 454-463, 1968.

Jaksic, T. and Burke, J. F. The use of "artificial skin" for burns. Annu. Rev. Med., *38:107-17*: 107-117, 1987.

Lotz, B. and Colonna, C. F. The chemical structure and the crystalline structures of *Bombyx mori* silk fibroin. Biochimie, *61*: 205-214, 1979.

Menegazzi, M., Guerriero, C., Carcereri, d. P., Cardinale, C., Suzuki, H., and Armato, U. TPA and cycloheximide modulate the activation of NF-kappa B and the induction and stability of nitric oxide synthase transcript in primary neonatal rat hepatocytes. FEBS Lett., *379*: 279-285, 1996.

Minoura, N., Aiba, S., Gotoh, Y., Tsukada, M., and Imai, Y. Attachment and growth of cultured fibroblast cells on silk protein matrices. J. Biomed. Mater. Res., *29*: 1215-1221, 1995.

Minoura, N., Aiba, S., Higuchi, M., Gotoh, Y., Tsukada, M., and Imai, Y. Attachment and growth of fibroblast cells on silk fibroin. Biochem. Biophys. Res. Commun., *208*: 511-516, 1995.

Minoura, N., Tsukada, M., and Nagura, M. Physico-chemical properties of silk fibroin membrane as a biomaterial. Biomaterials, *11*: 430-434, 1990.

Ohshima, Y. and Suzuki, Y. Cloning of the silk fibroin gene and its flanking sequences. Proc. Natl. Acad. Sci. U.S.A., *74*: 5363-5367, 1977.

Pandit, M. W., Sagar, A. J., and Rao, M. S. Studies on silk fibroin. I. Molecular weight, sedimentation coefficient, viscosity and optical rotation of silk fibroin from carbonate-extracted silk fiber. Arch. Biochem. Biophys., *149*: 259-268, 1972.

Purdue, G. F., Hunt, J. L., Still, J. M. J., Law, E. J., Herndon, D. N., Goldfarb, I. W., Schiller, W. R., Hansbrough, J. F., Hickerson, W. L., Himel, H. N., Kealey, G. P., Twomey, J., Missavage, A. E., Solem, L. D., Davis, M., Totoritis, M., and Gentzkow, G. D. A multi-center clinical trial of a biosynthetic skin replacement, Dermagraft-TC, compared with cryopreserved human cadaver skin for temporary coverage of excised burn wounds. J. Burn. Care Rehabil., *18*: 52-57, 1997.

Soejima, K., Nozaki, M., Sasaki, K., Takeuchi, M., and Negishi, N. Treatment of giant pigmented nevus using artificial dermis and a secondary skin graft from the scalp. Ann. Plast. Surg., *39*: 489-494, 1997.

Sprague, K. U. The *Bombyx mori* silk proteins: characterization of large polypeptides. Biochemistry, *14*: 925-931, 1975.

Whitfield, J. F., Isaacs, R. J., Jouishomme, H., MacLean, S., Chakravarthy, B. R., Morley, P., Barisoni, D., Regalia, E., and Armato, U. C-terminal fragment of parathyroid hormone-related protein, PTHrP-(107- 111), stimulates membrane-associated protein kinase C activity and modulates the proliferation of human and murine skin keratinocytes. J.Cell Physiol, *166*: 1-11, 1996.

DESCRIPTION OF THE INVENTION

The first aim of this invention is to overcome the problems and limitations of the known technique by proposing the use of a natural material as such or combined with biodegradable and non-biodegradable materials, of synthetic or natural origin, as the substrate suitable for the development, proliferation and correct differentiation of specialised tissue cells of the human body. This material should be biocompatible and stable or bio-resorbable in predefined times, in such a way as to obtain tissues, structures and organs that can be transplanted and/or implanted in or externally connected to the human body with their partial or complete structural and/or functional

integration therein or thereout.

The second aim of this invention is to propose the use of a substrate material that is suitable for the development of the above mentioned cell types, which can be produced easily and rapidly even on a large scale.

The most important features of this invention are described in detail in the main claim.

The subordinate claims describe particularly advantageous realisation forms of this invention.

The aforesaid aims are achieved thanks to the use of natural fibroin — e.g. obtained from, but not exclusively from the *Bombyx mori* silkworm, and also appropriately worked upon and/or mixed and/or co-polymerised and/or covering substances of natural and/or synthetic origin — as a substrate that is bio-compatible and bio-sorbable in predefined time lags and that is suitable to promote the survival, the proliferation, the maturation, and the differentiation of cells forming specialised tissues or organs of the human body, i.e. tissues consisting of cells with a high degree of differentiation, e.g. such as, but not limited to those making up the skin.

The cells whose survival, proliferation and differentiation is promoted by the substrate obtained from fibroin in accordance with this invention can give rise to both two and three-dimensional structures, according to the form of the substrate matrix on or in which they are cultured.

It is in fact known from the Literature that cells that have to proliferate on a three-dimensional scaffold, when appropriately stimulated by the addition of suitable factors, find themselves in a condition very similar to their natural one, as long as they are provided with a proper substrate.

According to another particularly advantageous aspect of this invention, the substrate obtained as per this invention, appropriately worked upon and/or mixed and/or co-polymerised, is particularly suitable to favour the survival, the proliferation, the maturation and differentiation of normal human adult osteoblasts originating from both the maxillary bones and the other bones of the skeleton, of mesothelial cells isolated

from the normal human adult peritoneum, of normal astrocytes isolated from the cortex of the human adult temporal lobe, of hepatocytes and liver stem cells, of liver biliary epithelium, of tenocytes (or tendon fibroblasts), of chondrocytes (or cells isolated from cartilaginous tissues), of endothelial cells of the *tunica intima* of blood vessels, of steroid-secreting cells of the adrenal cortex, of smooth muscle cells of the *tunica muscularis* of the intestine and blood vessels, of squamous epithelium of the oral cavity and the conjunctiva/cornea and of human pre-adipocytes of the white adipose connective tissue.

Detailed studies were carried out on the interactions taking place between the fibroin bio-membrane according to this invention and normal human adult dermal fibroblasts.

A series of additional tests were performed on the interactions of the fibroin membrane as per this invention with the main cell type found in human dermal connective tissue. Strains of normal fibroblasts obtained from samples of dermis taken from four different individuals were isolated in *in vitro* cultures. These cells were then seeded on samples of the bio-membrane as per this invention, contained in the rectangular wells of *in vitro* culture plates, and the fibroin bio-membranes holding the cells were incubated at 37°C for three weeks.

At predetermined time intervals the culture medium was sampled to evaluate the cellular consumption of glucose and the production of lactic acid. Moreover, in order to obtain more precise information about the events occurring at the interface between the human fibroblasts and the fibroin bio-membrane as per this invention, samples of this bio-membrane that had been in contact with the cells for predefined time lengths were examined, after removal of the fibroblasts without damaging the membrane, under a scanning electron microscope (SEM), an instrument that in expert hands reveals the finest details of the surfaces of the materials examined.

The results of these studies demonstrate that the human fibroblasts obtained from samples of dermis taken from four different individuals and seeded on the fibroin bio-membrane as per this invention not only multiply very actively , as was to be expected (data not reported), but at the same time also consume increasing amounts of

glucose, the favourite cell fuel, simultaneously secreting ever-increasing amounts of lactic acid, the metabolite of anaerobic glycolysis, into the growth medium. The *cumulative* curves of the consumed glucose and of the produced and secreted lactate have a fairly similar exponential trends which are in agreement with a simultaneous and quite remarkable expansion of the population of human connective tissue cells on the fibroin bio-membrane as per this invention. These curves also demonstrate that, albeit within the framework of an acceptable biological variability (human subjects are not inbred), once seeded on the bio-membrane, the fibroblasts isolated from four different individuals behave accordingly.

Thus, having further tested fibroblasts derived from five different individuals over two six-month periods, it is reasonable to assume that the bio-material as per this invention can establish very good biological interactions with the fibroblasts of most human subjects. Therefore, it seems to be particularly suitable as a substrate for tissue reconstruction in the clinical setting.

The SEM studies carried out on our specimens revealed a series of very interesting details on the nature of the interactions taking place between the human dermal fibroblasts and the fibroin bio-membrane as per this invention. When there was no contact with the human connective tissue cells, the surface of the bio-membrane appeared spotted in a totally casual way, with very small rises and hollows (Figure 10). After a week's contact with the human fibroblasts and once these had been appropriately removed, the surface of the bio-membrane appeared very different, exhibiting a series of grooves, mostly parallel to each other and with small pits or niches, as shown in Figure 11 (marked by arrows). During the first few days of *in vitro* culture, the cells evidently "dig burrows" in the bio-membrane, presumably thanks to the secretion of lytic enzymes in the extracellular environment. After two weeks of contact with the fibroblasts and once these had been removed, the "niches" in question were no longer visible on the surface of the bio-membrane even when observed at greater magnification (Figure 12). The surface of the membrane no longer presented niches, but protuberances, some small, others large (Figure 12, arrows). It is obvious that in the meantime the cells had produced and secreted into their environment the

components of the amorphous extracellular matrix which had filled the "niches" and accumulated onto the adjacent surfaces. Thus, it appears that, after an initial phase of "settling" on the fibroin bio-membrane, the human fibroblasts start to synthesise the materials necessary to reconstruct an environment that is familiar to them, i.e. the amorphous extracellular matrix of the dermal connective tissue. In slightly less scientific terms, the human cells do all they can to "make themselves comfortable". By observing Figure 13 it can be noted that after three weeks of contact with the fibroblasts and once these had been appropriately removed, the surface of the bio-membrane had taken a glassy appearance, since a homogeneous and "translucent" material covered all its irregularities.

SEM images also show that bundles of fibres with a slightly undulating pattern protrude more or less above this glassy surface; these are presumed to be bundles of newly formed collagen fibres shown by arrows in Figure 13. Thus, once adequate amounts of amorphous extracellular matrix had been formed, the fibroblasts produced and extra-cellularly secreted the precursors of the collagen fibres, which were then assembled together as normally occurs *in vivo*.

The *de novo* production of collagen fibres indicates that the human fibroblasts placed in contact with the fibroin bio-membrane as per this invention not only actively proliferate but also reach a fairly high level of differentiation and functional maturation.

Finally, immunohistochemical studies showed that, as occurs also *in vivo*, the protein vimentin constitutes the bulk of the intermediate filaments making up the cytoskeleton of the human fibroblasts cultured on the fibroin bio-membrane.

In brief, these observations convincingly demonstrate that the fibroin bio-membrane as per this invention constitutes a scaffold capable of interacting almost perfectly, from a biological point of view, with normal adult human fibroblasts, allowing them not only to proliferate actively but even to mature functionally.

The end result is that the fibroin bio-membrane as per this invention becomes inhabited by a population of normal mature human fibroblasts that produce and externally secrete both the components of the amorphous extracellular matrix and the precursors of collagen fibres (and perhaps elastic fibres too), just as occurs in normal *in*

vivo connective tissue.

In conclusion, from a biological standpoint the fibroin bio-membrane as per this invention has proven itself to be a substrate suitable to act as a scaffold for the creation of a bio-artificial dermis apt for clinical applications.

Furthermore, in order to experimentally validate the suitability the fibroin bio-membrane as per this invention also as a scaffold for a composite, dermo-epidermal system, studies were carried out on the interactions occurring between the bio-membrane according to this invention and normal adult human keratinocytes (i.e. epidermal cells).

As seen earlier, keratinocytes form the most superficial epithelial layer, usually known as the epidermis, of the skin. By definition, keratinocytes are epithelial cells since, at neat variance with fibroblasts, they adhere to each other very closely by means of membranal specialisations, so that the extracellular space between two adjacent epidermal cells is minimal. Furthermore, the epidermal cells produce and outwardly secrete numerous cytokines through which they intensely communicate with the adjacent keratinocytes via receptorial signalling systems. *In vivo* only the the *stratum germinativum* of the epidermis, i.e. the layer in close contact with the basal lamina (which marks the boundary with the connective tissue) contains precisely spaced stem cells, from the division of which and of their progeny the keratinocytes proper derive. In the remaining layers of the epidermis the keratinocytes undergo a complex and co-ordinated set of subcellular processes leading to their progressive differentiation and apoptosis ("*diffpoptosis*"), at the end of which they are eventually transformed into lifeless horny scales ("corneocytes"), which are still capable of mechanical protection and water loss prevention. Human keratinocytes can be cultured *in vitro*, but they require a continuous contribution of exogenous growth factors that *in vivo* are partly produced by the fibroblasts lodged in the underlying dermis, partly secreted by the keratinocytes themselves, and partly derived from the blood circulating in the dermal capillaries. The ability to reproduce at least a good part of the interactions that occur *in vivo* between the keratinocytes and the dermal fibroblasts would constitute the main advantage of clinically using dermo-epidermal substitutes (sheets of artificial skin) with

respect to grafting sheets only consisting of keratinocytes.

The keratinocytes isolated by means of enzymatic dissociation from the epidermis of three different subjects were seeded on portions of the fibroin bio-membrane according to this invention *(1)* alone, *(2)* in the presence on the same surface of human fibroblasts that had been previously irradiated (i.e. incapable of proliferative activity but still alive and functioning at least for a certain time), and *(3)* in the presence on the opposite side of the bio-membrane of human fibroblasts that had or had not been previously irradiated.

It should be remembered that the aim of the co-culture with the fibroblasts was to recreate a situation similar to that *in vivo*, in which the two types of cells are subjected to reciprocal interactions. The *in vitro* culture of the bio-membranes as per this invention seeded with keratinocytes with or without fibroblasts was carried on for at least three weeks.

The results obtained demonstrate for the first time that normal human keratinocytes seeded on samples of the fibroin bio-membrane in the absence of fibroblasts promptly adhere to it, forming fairly small cellular clumps. Three days after seeding, the keratinocytes exhibit a typical epithelial appearance and are possessed with normal cytological features. However, the absence of the fibroblasts makes itself felt, since the keratinocytes grow rather slowly and even nine days after seeding the cellular islets are a long way from confluence (Figure 16). These observations demonstrate that the fibroin bio-membrane allows for the adhesion, survival and growth (albeit a slow growth) of the normal adult human keratinocytes: an extremely encouraging preliminary result.

Even after seeding onto one of the surfaces of the bio-membrane previously covered in part by pre-irradiated (not proliferating but alive) human fibroblasts, the human keratinocytes promptly adhered to the substrate. Three days after seeding, numerous keratinocytes were present, forming fairly extensive islets between which the tapered forms of the fibroblasts could still be identified; even in this case the cytologic features of the keratinocytes appeared normal (Figs. 17 and 18). Nine days after seeding, the initially noted difference with the bio-membranes seeded with

keratinocytes alone was even more evident: the keratinocyte islets were much larger, while most of the pre-irradiated (incapable of replicating) fibroblasts had disappeared (Fig. 19). After 15 days, the pre-irradiated fibroblasts had completely disappeared, while the keratinocyte islets had merged together to form a continuous layer of cells, nearly all of which were still very small and, therefore, still actively proliferating (Fig. 20). On the other hand, human keratinocytes co-cultured with pre-irradiated fibroblasts on a *plastic surface* had formed after nine days moderately sized islets, which were on average considerably smaller than the islets of the same age on the fibroin bio-membrane as per this invention. Moreover, again on a *plastic surface* part of the epidermal cells had in the meantime grown considerably in size, thereby abandoning their proliferative activity to irreversibly move along the "*diffpoptosis*" pathway. Therefore, the simultaneous presence on the fibroin bio-membrane of pre-irradiated human fibroblasts is capable of significantly stimulating the proliferative activity of the human keratinocytes thereby preventing the onset of the *diffpoptosis* phenomenon. Thanks to this the human adult keratinocytes rapidly multiply, thus reconstituting in just 15 days a continuous structure similar to the germinative layer of the *in vivo* epidermis.

Finally, even when seeded on the surface opposite to the one to which the either pre-irradiated or untreated human fibroblasts had adhered, the human keratinocytes had formed numerous clearly distinct islets already after three days, consisting of small proliferating cells endowed with normal cytological features. Within nine days these islets had expanded considerably and after 15 days they had formed a continuous layer of cells that were still all small and thus actively proliferating. It can be concluded that if the fibroblasts are present even on the surface opposite to that occupied by the keratinocytes, and thus with no direct intercellular contact intervening between the two cell types, they keep producing powerful mitogenic stimuli for the epidermal cells.

Taken together these results clearly demonstrate that the simultaneous presence on the two opposite sides of the fibroin bio-membrane of the two main types of skin cells, i.e. keratinocytes and fibroblasts, is not only technically possible but leads also to the establishment of positive reciprocal interactions which, by effectively preventing the processes leading to *diffpoptosis*, induce the keratinocytes to proliferate intensely and to

form in approximately two weeks the equivalent of the *stratum germinativum* of the *in vivo* skin.

It is therefore apparent that the fibroin bio-membrane as per this invention is a nearly ideal scaffold for the production of sheets of bio-artificial skin, in which both keratinocytes and human fibroblasts can be simultaneously present and active.

ILLUSTRATION OF DRAWINGS

Further features and advantages of this invention will be evident from reading the following description of one form of realisation given here as an example, with the help of the figures enclosed herein, in which:

- Figure 1 shows a light microscope picture of the fibroin bio-membrane obtained in accordance with this invention, on the 12th day of staying *in vitro* in the total absence of human cells. The fine folds that increase the surface area of the membrane are particularly evident;
- Figure 2 shows normal human adult fibroblasts cultured *in vitro* and observed under the fluorescence microscope after staining with the fluorochrome acridine orange, which binds to nucleic acids. A metaphasic plaque of chromosomes in a dividing cell can be easily seen at the centre of the illustration;
- Figure 3 shows normal human adult fibroblasts cultured on the fibroin bio-membrane obtained in accordance with this invention, three days after seeding. Groups of cells close to each other can be noted;
- Figure 4 shows normal human adult fibroblasts in culture on the membrane obtained in accordance with this invention, six days after seeding. Cell accumulation and the two very fine folds that cross the bio-membrane along the horizontal axis can be observed;
- Figure 5 shows a light microscope picture of human fibroblasts in culture on the bio-membrane as per this invention, 21 days after seeding. The high density achieved by the monolayer of cells and the formation of a notable cellular aggregate consisting of several layers, visible on the right of the figure, are worthy of note;
- Figures 6 and 7 are graphs respectively illustrating the accumulation of urea and

of lactate in the growth media to which the normal human fibroblasts cultured on the bio-membrane as per this invention were exposed;

- Figures 8 and 9 are two diagrams of the cumulative curves of the consumption of glucose and the accumulation of lactate produced and secreted by normal human adult dermal fibroblasts taken from four different subjects, during 21 days of *in vitro* culture on the bio-membrane prepared according to this invention;

- Figures 10, 11, 12 and 13 show the ultrastructural changes undergone by the surface of the bio-membrane as per this invention. The bio-membrane is shown as it appears prior to cell seeding and after one, two and three weeks of contact with the normal adult human fibroblasts. The images were obtained with a scanning electron microscope (SEM), after removal of the fibroblasts by means of a technique that does not damage the bio-membrane in any way;

- Figures 14 and 15 both show light microscope pictures of normal human adult keratinocytes seeded on a bio-membrane obtained in accordance with this invention and cultured *in vitro* in the absence of fibroblasts, respectively showing their typical epithelial appearance and their normal cytological characteristics;

- Figure 16 shows a light microscope picture of normal human keratinocytes seeded on a bio-membrane obtained in accordance with this invention and cultured *in vitro* in the absence of fibroblasts, 9 days after seeding. Cell islets still a long way from confluence can be seen.

- Figures 17 and 18 show two light microscope pictures of normal human adult keratinocytes three days after seeding on a substrate consisting of a bio-membrane as per this invention, which had previously been partially covered with pre-irradiated (6000 Rad) human fibroblasts; the normal cytological features of the fibroblasts can be seen in Figure 18;

- Figures 19 and 20 show the normal human adult keratinocytes respectively 9 and 15 days after seeding on the substrate obtained as per this invention, previously inoculated with pre-irradiated fibroblasts that appear scattered;

- Figures 21 and 22 show normal human adult keratinocytes co-cultured with pre-irradiated fibroblasts on a standard plastic surface, nine days after seeding of the

keratinocytes; the cytological features of the cells can be seen in Figure 22;

- Figures 23 and 24 show two light microscope pictures of the surface of the substrate obtained as per this invention, this surface being opposite to the one on which normal human adult pre-irradiated and not pre-irradiated fibroblasts had been previously inoculated, three days after seeding;

- Figure 25 is a light microscope picture of what is described in Figures 23 and 24, 9 days after seeding of the keratinocytes;

- Figure 26 is a light microscope picture of what is described in Figure 23, 15 days after seeding of the keratinocytes.

DETAILED DESCRIPTION OF THE INVENTION

Information will now be given regarding fibroin, the experimental method used to obtain it and the tests carried out on three types of membrane in order to verify the effectiveness in achieving the aims of this invention, the results of which made it possible to determine the most suitable type of membrane.

Details will then be given concerning the methodological procedures employed for the *in vitro* culture and the proliferation assay of specialised tissue cells of various types of the human body.

The following paragraph will therefore, in certain detail, describe fibroin, a natural protein contained in silk, thus produced by the domestic silkworm *Bombyx mori*, and currently also obtainable by means of genetic engineering techniques. The use of this material as a substrate for the survival, proliferation maturation, and differentiation of tissue cells, differentiated or otherwise, of the human body, constitutes the grounds for the patentability of this invention.

The silky filament of the domestic silkworm consists of two monofilaments of fibroin, a fibrous protein, cemented together by sericin, a gelatinous protein.
Fibroin is formed by a sequence of amino acid residues consisting mainly of glycine (gly), alanine (ala), serine (ser) and tyrosine (tyr).

These amino acids can account for up to 90% of the entire molecule.

In short, the structure of the crystalline component precipitated from raw fibroin, as identified after incubation with chemotrypsin, and constituting the fraction

known as Cp, is:

Gly-Ala-Gly-Ala-Gly-Ser-Gly-Ala-Gly-[Ser-Gly-$(Ala-Gly)_n$]$_8$-Tyr

The remaining 10% is an irregular mixture in terms of composition and sequence of almost all the remaining amino acids, including low amounts of proline and hydroxy-proline, while cystine is absent or nearly so.

These features distinguish fibroin, for example, from keratin and from collagen, and explain some of its contrasting mechanical properties, particular importance being given to its inextensibility, whatever the environmental conditions in which it finds itself.

The molecular weight of the macromolecule is 370 kD and is formed by a chain known as L, i.e. light, weighing 25 kD, and a larger chain known as H, i.e. heavy, weighing 350 kD. These two chains are joined together by a disulfide bridge.

Fibroin presents in three different possible phases:

- the amorphous phase, also known as "random coil", which is soluble in water;
- the α-helix phase, also known as "silk I", which is metastable and also soluble in water, and which, as such, is easily subject to a phase transition towards the β-sheet phase, also known as
- "silk II", which is not hydro-soluble and is formed by parallel chains joined together by hydrogen bonds with a high order level.

The natural transition of any system from a metastable form to a stable form, in this specific case from the "silk I" form to the "silk II" form, takes a very long time, since it is merely the result of the reorganisation of each individual atom in the constituent molecules from a high energy state to a lower, and hence more stable, energy state.

This is why nature ensured that this transition occurred during the silkworm's secretion phase. An analysis of the fibroin secreted by the silkworm reveals the presence of a 55% of crystals in the ß-sheet form, which are stable and scattered in an amorphous matrix; obviously, the reverse process only is possible via protein degradation.

By dissolving the fibroin it is also possible to obtain a totally amorphous material, therefore entirely soluble in water.

The complex secondary molecular structure, where this refers to the development of the molecules in the three-dimensional space, of silk can be used to check specific interactions in different chemical and mechanical environments.

The presence and frequency of the various crystalline structures and of the amorphous zones in the fibroin molecule can be modified by stretching, by compression or by means of various chemical methods. The aims of these processes that lead to conformational changes consist in the preparation of stable membranes acting as barriers and substrates to immobilise and store enzymes. See on this topic A. Kitamura et al. Application of silk fibroin to functional membranes: relationship between conditions of membrane preparation, membrane potential and ion permeability. Published in Sen-I Gakkaishi, vol. 44, no. 4, 1988, pages 193-197.

The invention described here is not limited by the following accomplishment examples.

The Literature clearly shows that the conditions required for the preparation of the fibroin membrane by means of *casting*, in other words the concentration, the solvent, the substrate, the temperature, etc., are all linked to the properties of the resulting fibroin membranes. These properties are the degree of crystallinity, the absorption of water and the conformation. On the basis of this relationship, the various preparation parameters were studied in order to obtain a material that overcomes the initial problems of fragility and instability in a watery environment that are typical of such a material, as seen above.

Once perfected, the preparation conditions and procedures were standardised to make them totally reproducible and to obtain samples that are always homogeneous.

Obtaining the fibroin

The fibroin is obtained through a normal process of degumming:

- raw silk (fibroin and sericin) in fabric form was placed in a watery solution of Marseilles soap at a concentration of 10 g/l with a fibroin bath/soapy solution of 1:100, at a temperature of 98°C for an hour. The samples were then washed repeatedly in

distilled water and any impurities and fatty substances were extracted in petroleum ether.

The efficiency of the degumming process was checked by observing under the light microscope a sample stained with *Coomassie blue* stain.

Preparation of the fibroin membranes

The procedure for the preparation of the fibroin membranes is based on protocols already existing in the Literature, such as for example the procedure defined by N. Minoura et al. in their article entitled: Physico-chemical properties of silk fibroin membrane as biomaterial. Published in Biomaterials, Vol. 11, August 1990, pages 430-434. These protocols were appropriately modified in order to perfect the level of biological interaction of the membranes with the cellular environment with which they are placed in contact.

The degummed silk obtained as described in the previous example was dissolved in a watery solution of 9 M of lithium bromide at a weight/volume ratio of 1 g/10 ml with a 10% solution, at a temperature of 65°C for 3 hours.

The solution obtained was filtered through a no. 1 porous septum and diluted with distilled water to bring the solution to a 5% weight/volume ratio.

The solution was dialysed against water for 2 days in order to achieve the total elimination of salt.

Spectra/Por dialysis tubes manufactured by Spectrum - MWCO: 3,500 were used, 14 mm in diameter and with a volume/length ratio of 1.0 ml/cm. The dialysis membrane selected had a low molecular cut in order to retain small peptides in the solution, that became detached during the previous phases and which seem to favour cell growth.

Casting of the watery solution at 5% (w/v) was performed at room temperature with a polystyrene mould.

This polymer was chosen for the mould since it was found not to interfere with the structure of the material (formation of a material with a high percentage of crystallinity on the glass), and also because it does not modify the surface morphology of the membrane in contact (as polyethylene does).

The product obtained is an amorphous state membrane, thus completely soluble in water. Transition from the random-coil form to the required stable and orderly β-sheet form was achieved by treating the membrane with a watery solution of methanol (80:100) by means of a process already known in the Literature and described by N. Minoura et al. in "Fine structure and oxygen permeability of silk fibroin membrane treated with methanol", Polymer , Vol. 31, February 1990, pages 265-269.

Evaluation of the stability of the materials treated in a watery solution of methanol

This was performed by assaying the proteins released in a saline solution (PBS), evaluating the molecular weights by HPLC analysis.

The materials used for comparison are a control material, an untreated, or amorphous, material and a material physically treated with heat.

Sample	Weight (mg)	Incubation in PBS (ml) for 1 hour	Proteins released (mg/ml)
Control	4.5	1.2	0.458
200°C	5.2	1.2	0.401
Methanol (80%)	4.7	1.2	0.177

The table shows that the treatment with methanol is more effective in inducing a change in protein conformation and in inducing it to acquire the β-sheet conformation, which makes the membrane extremely stable in water since it causes the establishment of strong intermolecular hydrogen bonds.

Characterisation

The characterisation parameters of the fibroin molecule obtained by means of the procedure described in the preceding examples are as follows:

a) molecular weight of the degummed fibre and of the fibroin after the solubilisation and dialysis phases;

b) molecular weight of the lyophilised dry residue released in distilled water at different times by membranes obtained with different preparation methods;

c) angle of contact;

d) water absorption measurements;

e) physical-mechanical tests and dynamic mechanical thermal analysis (DMTA);

f) Differential Scanning Calorimetry

Determination of the molecular weight of fibroin in steric-exclusion chromatography

The fibroin membrane samples were analysed by means of chromatography, with the following instruments:

- HPLC Waters consisting of a model 510 pump, a U6K injector, a UV/Vis model 490 detector. The analysis conditions and the data acquisition and processing were handled by the Maxima 820 chromatographic software;

- Shodex Protein KW-804 column.

Fibroin

Analysis of the results does not reveal any significant differences in the distribution of the molecular weights between the fibroin samples analysed (fibre after degumming and fibroin after solubilisation with LiBr).

In both cases, the denaturating conditions of the mobile phase led to a great increase in the hydrodynamic volume of the fibroin molecules, placing them at the upper limits of the fractioning range of the column used (average apparent molecular weight: 650-700 kD). This is demonstrated by the asymmetrical form of the elution curve, which presented a rapid ascent in correspondence with the exclusion volume of the column, followed by a gradual descent towards the region of low molecular weights. Nor can it be excluded that intermolecular aggregation phenomena occur in the solution.

However, from the results obtained it can be concluded that solubilisation with lithium bromide does not appear to have led to significant modifications to the structure of the fibroin, the molecular weight being practically unchanged with respect to the initial fibre.

Residues

The protein material that was detached from the films preserved in water for the times adopted is characteristic, from a chromatographic point of view, since it contains

mainly protein fragments with a medium-low molecular weight.

It is believed that these are fragments of fibroin, in particular peptides originating from the low molecular weight subunit L (Light chain), which were detached from the film immersed in water as the result of the interaction with various factors that act in a concentrated way, such as the swelling action of the water and extraction-solubilisation of the more soluble protein fractions, the probably hydrolytic action of the water, the probable variations in pH and the fragments that are detached from the chain during the phase of fibroin solubilisation in lithium salts.

In fact, the alkaline conditions of degumming can induce partial hydrolysis of the disulfide bridge that in natural fibroin binds the subunits H (Heavy chain) and L. This hypothesis is reinforced by the presence of a shoulder that emerges in the low molecular weight region of the fibre elution curve, as well as by previous experience (unpublished data).

Angle of contact

The dynamic angle of contact (advancing and receding) was measured using a Cahn model 322 microbalance and the Wilhelmy technique. Moist samples (steady state) measuring 20x5x0.15 mm were analysed with 3 cycles of immersion and emergence at 150μm/sec in distilled water by HPLC (Merck) at a temperature of 25°C.

The samples in question did not show any appreciable phenomena of hysteresis, and the average angle of contact measured was 55° ± 3°.

Samples comparable to the previous ones but with a lower degree of water absorption showed much higher angles of contact with respect to the value reported above.

It should in any case be pointed out that the experimental value to be considered is the one measured on fibroin membranes in a steady state, since it reproduces a more correct physiological situation.

Absorption tests in distilled water

The membranes were weighed after stove drying at 60°C and then at increasing incubation times in distilled water (15, 30, 60 minutes, 2 hours, 4 hours, 8 hours and

from one to 45 days).

Maximum water absorption is evaluated at around 40% in weight after 2 days in a watery environment.

Mechanical tests

Measurements were taken relative to the mechanical characteristics of the membranes at different degrees of hydration.

Incubation	Module (MPa)	Max Stress (MPa)	Extensibility %
5 hours	27.6± 4.3	3.3 ± 0.1	79 ± 12
10 days	58.6 ±15.4	4.0 ± 0.3	60 ± 18
20 days	114.1 ± 2.3	5.9 ± 0.1	80 ± 35
30 days	111.9 ± 12.4	5.7 ± 0.3	71 ± 18

The curves seem to show an increase in the resistance of the material as the water absorption increases.

It is possible that there is a loss of substances with a plasticising effect and/or the formation of inter-chain bonds that confer to the membrane superior mechanical characteristics, with a simultaneous structural variation in favour of the β-sheet or silk II crystalline phase.

Comparative test

Three membranes were produced, two of which, membranes B and C, made

from non-purified fibroin thus containing negligible fractions of impurities, and membrane A, consisting of a copolymer of D, L-lactic acid at 60% in weight and of ε-caprolactone at 40% in weight, and each of them proved to be suitable for use as a substrate in accordance with this invention.

The procedures employed to obtain the three membranes are described below.

Membrane A

D,L-lactic acid (Boehringer-Ingelheim) was purified by recrystallization from a solution in ethylacetate and dried at 45°C under vacuum for 24 hours.

The ε-caprolactone (Aldrich) was distilled under reduced pressure and preserved under nitrogen pressure.

The polymer was produced by causing the monomers to react in nitrogen atmosphere, using stannous ethyl-hexoate as a catalyst, in a glass reactor agitating at 120°C. After polymerisation, the copolymer was purified by dissolution in acetone and subsequent precipitation in methanol. The membrane was prepared by solvent evaporation from a solution of the copolymer purified in acetone, subsequently eliminating the solvent by oven vacuum treatment.

Membrane B

The degummed silk was dissolved in a 9 molar solution of lithium bromide in water, at a temperature of 65°C for three hours, at a concentration of 0.1 g/ml.

The solution was then filtered through a porous ceramic filter and diluted with distilled water until it reaches a concentration of 0.05 g/ml, and then dialysed against water for two days to eliminate the salt, using dialysis tubes (for example Spectra/Por manufactured by Spectrum – MWCO = 3500, diameter = 14 mm, Volume/Length = 1.5 ml/cm).

The solution was then poured into polystyrene containers and the membrane was obtained by evaporation of the water at room temperature.

The membrane was then immersed in a solution of 80% methanol in water for 60 seconds to render it crystalline and thus insoluble in water.

Membrane C

The degummed silk was dissolved in a 9 molar solution of lithium bromide in water, at a temperature of 65°C for three hours, at a concentration of 0.1 g/ml.

The solution was then filtered on a porous ceramic septum and diluted with distilled water until it reaches a concentration of 0.05 g/ml, and then dialysed against water for two days to eliminate the salt, using dialysis tubes (for example Spectra/Por manufactured by Spectrum – MWCO = 3500, diameter = 14 mm, Volume/Length = 1.5 ml/cm).

The solution was then brought rapidly to freezing temperature in liquid nitrogen and then freeze-dried.

The freeze-dried solution was dissolved in isopropanol fluoride, obtaining a 5% (weight/volume) solution of fibroin.

The solution was then poured into polystyrene containers and the membrane was immersed in a solution of 80% methanol in water for 60 seconds to make it crystalline and thus insoluble in water.

The three membranes were sterilised in ethylene oxide, placed in contact with normal human fibroblasts from previously informed adults, and incubated at 37°C in a growth medium for three weeks.

Observations were then made using an inverted microscope, both under normal and fluorescent light, of the *in vitro* cultures obtained, while the growth media were changed at regular intervals and the cell-conditioned media were sampled and stored at -80°C for subsequent biochemical analysis.

With particular reference to the Figures, it can be seen how the survival and proliferation of the normal adult human keratinocytes in the culture grown on the membrane B obtained in accordance with this invention is particularly evident, and Figures 17-25 also show how the prior seeding of pre-irradiated and non-pre-irradiated normal adult human fibroblasts, in order to prevent their proliferation, which takes place twice as quickly compared with the proliferation rate of the keratinocytes, did not prevent or in any way condition the crowding of the substrate as per this invention by the epidermal cells, even when they were seeded on the opposite surface of the membrane.

Moreover, the images clearly show how the cytological features of the cells making up the cultures grown on the surface of the substrate are completely normal.

The results obtained and the comparisons of membrane B as per this invention with the other two membranes tested for efficiency led to the following surprising conclusions:

membrane B as per this invention and membrane C

a) allow a more intense cellular growth with respect to membrane A;

b) allow very intense synthesis metabolic processes to take place without significantly activating proteocatabolic processes.

Moreover, membrane B as per this invention in comparison with membrane C

can be produced in considerable quantities at lower costs, thus having not only typical biological advantages, but even economic ones and thus also being easier to market.

Protein expression

Although the morphology of the keratinocytes and their ways of aggregating clearly differ from those proper of the fibroblasts and can be seen even by a non-experienced eye, it seemed appropriate to demonstrate that on the bio-membrane as per this invention they express specific cytoskeletal proteins proper of the intermediate filaments, i.e. the cytokeratins (not expressed by the fibroblasts).

Sheets of the bio-membrane obtained as described in Example X, on which keratinocytes had been cultivated, were challenged with a "*pan*" antibody directed against the cytokeratins and then incubated with a secondary antibody marked with FITC or bound to alkaline phosphatase. We thus could demonstrate that the keratinocytes contain cytokeratins filaments in their cytoplasm. On the other hand, an antibody directed against vimentin, the typical protein of the intermediate filaments of the fibroblasts, decorated the intermediate filaments of the latter cells very clearly without reacting with the keratinocytes in any way. The two cell types thus maintain cyto-specific characteristics even when cultured on the bio-membrane as per this invention.

IN VITRO CELL CULTURE METHODS

Example I

In vitro culture method of normal squamous epithelial cells of the human epidermis, oral cavity, conjunctiva and cornea and of fibroblasts of the underlying connective tissues

The above-mentioned cells can be isolated with comparable techniques from biopsy samples of the corresponding tissues (skin, oral mucosa, conjunctival mucosa and cornea). As an example for them all, the methods applied to the epidermis and the dermis obtained from human skin biopsies will be described.

On reaching the laboratory, the skin sample is incubated at 4°C overnight in a dispase solution (0.25% w/v). The attenuated enzymatic digestion makes it possible to detach the epidermis (as a single lamina) from the underlying connective tissue (dermis and subcutaneous tissue) with no difficulty; the two tissue samples obtained in this way undergo separate treatments.

(A)Dermis and subcutaneous tissue

The aim of the procedure is to isolate the fibroblasts, which are the cells assigned to maintain the structure of the connective tissue and which can be cultured separately or co-cultured with the keratinocytes (*'feeder layer'* method).

The connective tissue sample is cut into fragments thanks to the scissor-like action of two scalpel blades in a solution of trypsin (0.25% w/v) and EDTA (0.02% w/v); enzymatic digestion is then performed in an incubator at 37°C with the help of a magnetic stirrer maintained at low speed (50 rpm) for 30 minutes, followed by centrifugation at 600 rpm for 10 minutes a 4°C. The supernatant is decanted, the pellet is resuspended and the living cells are counted in a Neubauer chamber. The fibroblasts, pre-irradiated with gamma rays (6000 rad) or non-pre-irradiated, are then seeded.

(B) Epidermis

The aim of the procedure is to isolate the epidermal keratinocytes, which are the main type of epithelial cells of the skin. The thin epidermal lamina is carefully and quickly fragmented in a trypsin solution (0.25% w/v). A specific trypsin inhibitor is then added and the solution is centrifuged at 600 rpm for 10 minutes at 4°C. The supernatant is decanted, the pellet is resuspended and the living cells are counted in a Neubauer

chamber. The keratinocytes are then seeded.

Supports used for the* in vitro *cultures

1. Plastic flasks with treated or untreated surfaces to increase the adhesiveness

2. Plastic flasks with surfaces as above, coated or not with a layer of pre-irradiated fibroblasts

3. Fibroin membranes (pure or combined with other compounds) with the upper surface coated or not with a layer of normal fibroblasts or fibroblasts pre-irradiated with gamma rays (6000 rads)

4. Fibroin membranes (pure or combined with other compounds) with the lower surface (i.e. the one opposite the upper surface) coated or not with a layer of normal fibroblasts or fibroblasts pre-irradiated with gamma rays (6000 rads)

Culture medium

The medium 3MCDB153:1 (consisting of three parts of Dulbecco's Modified Eagle Medium [DMEM] and one part of Medium F12) is normally used, to which foetal bovine serum (FBS; 10% v/v), antibiotics (solution of penicillin-streptomycin 1% w/v), epidermal growth factor (EGF; 0.1 μg /ml), insulin (20 ng/ml), pituitary extract (PTE; 30 μg/ml) and hydrocortisone (0.5 μg/ml) are added. The medium is replaced every day or every other day with a fresh medium of the same composition.

Evolution of the cultures

The normal human fibroblasts proliferate rapidly, forming first a continuous monolayer of cells parallel to one another and then a series of layers with cells that are not always in parallel. In the meantime, the fibroblasts produce and secrete increasing quantities of extracellular matrix components and of collagen fibre precursors, the latter ones being then integrated in bundles of fibres which may even be quite large in size. The fibroblasts are avid consumers of the glucose present in the culture medium and, typically, secrete considerable quantities of lactic acid into the medium. The fibroblast cytoplasm contains both intermediate filaments consisting of vimentin and their cytotypical marker, decorin.

The normal human keratinocytes proliferate rapidly, starting from minute clusters and

form a single layer of small and highly adherent epithelial cells. They have a mitotic cycle time of about 48 hours and their cytoplasm contains intermediate filaments formed from the so-called "light" cytokeratins (while the cells are proliferating actively) or "heavy" cytokeratins (when the cells differentiate). If the keratinocytes reach the surface of the growth medium, they form a series of layers whose surface cells end up by entering the phase of terminal postmitotic differentiation (*diffpoptosis*). *Diffpoptosis* is triggered even if EGF is withdrawn from the cells for longer than 48 consecutive hours. The growth rate of the keratinocytes is much faster if human or rodent pre-irradiated or non-pre-irradiated fibroblasts are present on the bottom of the flask or on the reverse surface of the culture scaffold.

Example II

In vitro culture method of normal human adult astrocytes

Astrocytes are neuron supporting cells and can be isolated from intra-operative biopsy samples (e.g. the cerebral cortex of the temporal lobe). On reaching the laboratory, the nervous tissue biopsy is cut into minute fragments, subjected to a bland enzymatic digestion with trypsin (0.25% w/v) in Hanks' Basal Salt Solution (BSS) at room temperature (18°C) and, finally, mechanically dissociated by repeatedly triturating the tissue fragments with Pasteur pipettes endowed with progressively smaller bores. The isolated cells are then seeded in culture flasks where they proliferate in an *ad hoc* medium (see below). Initially their growth is very slow. Once they reach a 70% confluence, the nerve cells are detached with a solution of trypsin (0.25% w/v) and EDTA (0.02% w/v) in BSS and seeded into new flasks. The procedure is repeated several times over a number of months.

Supports used for* in vitro *cultures

1. Plastic flasks with treated or untreated surfaces to increase cell adhesiveness

2. Fibroin membranes (pure or combined with other compounds)

Culture medium

Dulbecco's Modified Eagle Medium (DMEM) is normally used, to which FBS (10% v/v), antibiotics (solution of penicillin-streptomycin 1% v/v), basic fibroblast growth factor (bFGF; 20 ng/ml), insulin-like growth factor-1 (IGF-1; 20ng/ml), platelet-derived

growth factor (PDGF; 20 ng/ml), epidermal growth factor (EGF; 10 nM), β-estradiol (10 nM) and cholera toxin (10 nM) are added. The medium is replaced every 2-4 days with a fresh medium of the same composition.

Evolution of the cultures

When actively proliferating, normal human adult astrocytes appear as small polygon-shaped cells with an epithelial-like appearance, which may subsequently differentiate, rather than continuing to proliferate, moving towards the phenomenon of *stellation*, i.e. the emission of numerous richly arborized cytoplasmic extensions. Changing the medium frequently prevents *stellation* from appearing and maintains an intense proliferative activity; changing the medium very rarely has diametrically opposed effects. The astrocytes do, however, express cyto-typical markers, such as *glial fibrillary acid protein* (GFAP) and the GAP-43 protein, which can be detected by immunohistochemistry and Western *immunoblotting*. In protracted cultures, human astrocytes form extremely intricate networks of finely arborized cellular extensions which are superimposed on one another in several layers.

Example III

***In vitro* culture method of normal squamous epithelial cells (mesothelial) of human adult serous membranes**

Human mesothelial cells are isolated by means of enzymatic dissociation from intra-operative biopsies of the serous membranes (pleura, pericardium, peritoneum). As an example for them all, we will refer to the procedures applied to a biopsy sample of greater omentum (peritoneum).

On reaching the laboratory, the biopsy fragment is thoroughly washed in a saline solution. Enzymatic digestion is then performed with a dissociating solution containing trypsin (0.125% w/v) and EDTA (0.02% w/v) in Hanks' Basal salt Solution (BSS). Digestion is carried out in an incubator (at 37°C) stirring the tissue slowly (40 rpm) for 25 minutes. The supernatant obtained is then centrifuged at 1700 rpm for 5 minutes at 4°C. This is followed by re-suspending, counting and seeding of the epithelial cells forming the pellet. Additional cycles of enzymatic digestion of the tissue remaining after the first dissociating cycle can be carried out . However, this involves the risk of

also isolating the omental fibroblasts rather than the mesothelial cells alone. At the end of these cell dissociation cycles, the remaining tissue is cut into minute fragments which are also inoculated into the culture flasks.

Supports used for the* in vitro *cultures

1. Plastic flasks with treated surfaces to increase cell adhesiveness

2. Fibroin membranes (pure or combined with other compounds)

Culture medium

The culture medium used to culture the human mesothelial squamous cells has the following composition: Ham's F12 enriched with FBS (10% v/v), antibiotics (solution of penicillin-streptomycin 1% w/v), insulin (0.5 µg/ml), glutamine (2 mM), iron-saturated human transferrin (0.5 µg/ml), hydrocortisone (0.4 µg /ml) and cholera toxin (10 ng/ml). The medium is renewed every 4 days with a fresh medium of the same composition.

Evolution of the cultures

The epithelial cells of the human peritoneal mesothelium have initially a small size and group together in tiny clusters sporadically interspersed by fibroblasts. The cells proliferate actively, generating polygon-shaped elements endowed with a cytoplasm rich in organelles that grows in size as time passes and ends up by undergoing a strong vacuolization. Among the increasingly larger and often binucleate epithelial cells, which form more and more extensive sheets but always remaining in a monolayer, in which some nests of small actively proliferating cells persist. These nests may join with one another thereby forming a network. The few initial fibroblasts do not proliferate but die due to apoptosis unless their growth is favoured.

Example IV

In vitro culture of normal adult human osteoblastic line cells

Intra-operative biopsy fragments of bone tissue from the maxillary bones or from other bones of the human skeleton are repeatedly washed in phosphate buffered saline solution A (PBS/A) before undergoing two pre-digestion cycles with type II collagenase (0.5 mg/ml) for 45 minutes at 37°C. The bone fragments are chopped with a bone cutter

and cultured in cluster-type plates with 12 wells each.

Supports used for* in vitro *cultures

1. Plastic flasks with treated or untreated surfaces to increase cell adhesiveness

2. Fibroin membranes (pure or combined with other compounds)

Culture medium

The growth medium used for the *in vitro* culture of the human osteoblastic cells has the following composition: Dulbecco's Modified Eagle Medium (DMEM) enriched with FBS (10% v/v), antibiotics (penicillin-streptomycin solution 1% w/v), sodium ascorbate (50 µg/ml) and borosilicate glass slivers. The medium is renewed every 4 days with fresh medium with the same composition. The addition of ß-glycerophosphate and agents such as vitamins A and D_3 to the above-mentioned medium exerts a differentiating effect (rather than a merely mitogenic one) on the osteoblastic cells.

Evolution of the cultures

After the first two weeks of staying in culture, the cells that migrate out of the bone fragments are detached by trypsinization (trypsin at 0.25% w/v) from the borosilicate glass slivers to which they mainly adhered and seeded into plastic culture flasks, again using DMEM enriched with FBS and ascorbate. After a further round of trypsinization, the cells can be placed onto any other type of support. The osteoblastic cells first form single layers in which the arrangement of the various elements leads to the creation of vortices which become more and more marked as the cells, progressively increasing in number, form several layers. The proliferating cells do not initially express their specific plasmalemmatic marker, i.e. the alkaline phosphatase enzyme. This appears at a subsequent stage and continues to be expressed with varying intensity by the several osteoblastic cells, paralleling their current degree of differentiation. The most mature cells practically stop proliferating but secrete the collagen fibre precursors and the extracellular matrix components, including osteocalcin and osteonectin, into the medium. Eventually, the calcification processes of the extracellular matrix, which are first focal and then more widespread, can be so intense as to lead to the *in vitro* formation of structures very similar to cancellous bone.

Example V

In vitro culture of normal neonatal rat liver hepatocytes

Neonatal rat hepatocytes are isolated by means of combined enzymatic and mechanical dissociation of the liver of animals not more than 4 days old. After the animals have been sacrificed using a painless technique, the livers are removed using a special surgical table that is cooled in order to limit the consumption of oxygen by the hepatic tissue to a minimum. Once extracted, each liver is chopped into pieces measuring around 2-3 mm^3. The pieces of tissue are washed several times in saline solution. Digestion is then carried out with a solution consisting of type II collagenase (5 mg/ml), ovine testicular hyaluronidase (5 mg/ml) and trypsin (0.02% w/v), in which the pieces of liver are slowly stirred for 15 minutes. At the end of the first round of enzymatic digestion, the liquid phase is removed and the activity of the enzymes is blocked by adding protease inhibitors and FBS (20% v/v) before storing it at 4°C. The rounds of enzymatic dissociation are repeated four-five times, storing the corresponding supernatants at 4°C. The pieces of liver "softened" by the incubation with the proteases are triturated several times with Pasteur pipettes of increasingly smaller bores. This mechanical action almost completely frees the cells from the connective tissue stroma. The fractions obtained by enzymatic and mechanical dissociation are mixed and centrifuged at 600 rpm for 5 minutes at 4°C. The cells of the pellet obtained by this procedure are resuspended and counted. Finally, the hepatocytes are separated from the stromal cells by using a Percoll™ gradient before seeding them onto the culture supports.

Supports used for the* in vitro *cultures

1. Plastic flasks with treated or untreated surfaces to increase cell adhesiveness

2. Fibroin membranes (pure or combined with other compounds)

3. Ultrathin (37.5 μm thick) porous discs of non-toxic polyethylene

Culture medium

The growth medium used for the neonatal rat hepatocytes is the $HiWo_5Ba_{2000}$ medium, to which FBS (10% v/v) or no FBS and antibiotics (cephaloridin-streptomycin solution 0.1% w/v) are added in the first few hours.

Evolution of the cultures

Using the Percoll™ cultures are set up in which the neonatal hepatocytes make up at least 98% of the total cell population. The cells group together in homogeneous islets in monolayer and, generally, already after 24-48 hours show the reconstitution of the biliary canaliculi. A small fraction (around 15%) of the hepatocytes proliferate spontaneously in the cultures and around 8 % of the cells die every day *in vitro* due to spontaneous apoptosis. This means that in basal conditions the *in vitro* hepatocellular population maintains an almost constant size or grows only slightly even for more than 30 days. Enriching the serum-free $HiWo_5Ba_{2000}$ with growth factors, such as *epidermal growth factor* (EGF), *transforming growth factor-α* (TGF-α), *hepatocyte growth factor* (HGF), pancreatic hormones (insulin and glucagon), phorbol esters and cyclic adenosine monophosphate 3',5' (cAMP) increases the size of the proliferating hepatocellular fraction and, at the same time, of the hepatocellular population as a whole. The growth of the few stromal cells present at the start in the cultures is prevented by using the selective medium $HiWo_5Ba_{2000}$ that does not contain the essential amino acid arginine, which the connective tissue cells are unable to synthesize. The neonatal rat hepatocytes produce *ex novo* cyto-specific markers, such as albumin, fibrinogen and other plasma globulins and secrete them into the growth medium as well as specifically uptaking ^{3}H-bilirubin from the medium, during four weeks of staying in *in vitro* culture.

Example VI

Culture of steroid-secreting cells of normal human adult adrenal cortex

Portions of normal adrenal tissue are taken, after obtaining informed consent, from adult patients during operations for the simultaneous removal of kidney and the adrenal gland on the same side. The glandular tissue is decapsulated to eliminate the *zona glomerulosa* and then placed in a Universal type container (volume = 25 ml) containing 10 ml of Eagle Minimum Essential Medium (MEM) enriched with FBS (20 % v/v) inactivated at 56°C for 30 minutes and, finally, sent to the laboratory in a Dewar flask containing melting ice. The procedures for the culture of the adrenal cortex tissue begin

within 30 minutes-6 hours of removal of the sample. The tissue is transferred to a Petri dish (diameter: 10 cm) containing 10 ml of cold (4°C), sterile *phosphate buffered saline* solution A (PBS/A). The tissue is cut into pieces 4-5 mm in size, which are repeatedly washed with sterile PBS/A to remove the blood cells, and finally transferred to a 50 ml Erlenmeyer flask containing a solution of trypsin (0.2% w/v), type I collagenase (0.5% w/v) and ovine testicular hyaluronidase (0.4 % w/v). The pH is adjusted to 7.3-7.4 by adding a small amount of $NaHCO_3$ solution (8% w/v). The histolytic mixture is heated to room temperature (18°C). The pieces of tissue are left to rest without any agitation in the disaggregating solution for 30 minutes. Then they undergo four subsequent rounds of magnetic stirring (100 ± 20 rpm) lasting 20 minutes each. The various supernatants are removed, mixed with protease inhibitors and inactivated FBS (20% v/v) and stored at 4°C. The remaining pieces of tissue are transferred to a Petri dish where they are chopped with two knives. The supernatants and the fragments of tissue are mixed together and centrifuged twice at 50 *g*. The cells in the remaining pellets are mixed together prior to seeding.

Supports used for the* in vitro *cultures

1. Plastic flasks with treated or untreated surfaces to increase cell adhesiveness

2. Fibroin membranes (pure or combined with other compounds)

3. Ultra-thin (37.5 μm thick) porous discs of atoxic polyethylene

Culture medium

The culture medium used is Eagle MEM, enriched with inactivated FBS (20% v/v), cephaloridine (50 μg/ml), penicillin and streptomycin (50 μg/ml) and nystatin (25 UI/ml). Alternatively the serum-free $HiWo_5Ba_{2000}$ medium is used with the addition of the above-mentioned antibiotics. The medium may also be enriched with a trophic-differentiative factor specific for the adrenal cortex cells, i.e. the adrenocorticotropic hormone (ACTH) in its entire (amino acids 1-39) or shortened form (amino acids 1-24).

Evolution of the cultures

In the absence of ACTH the cells of the *zonae fasciculata* and *reticularis* of the adrenal cortex resemble fibroblasts, lose many of their typical ultrastructural and functional

features and may move towards apoptosis. The addition of ACTH to the medium stimulates the proliferative processes and leads to marked effects of ultrastructural differentiation (proliferation of the smooth endoplasmic reticulum, differentiation of the tubulo-vesicular *cristae* of the mitochondria, hypertrophy of the Golgi apparatus, and reduction of the size of lipid droplets) and to an intensification of the steroidogenetic processes and of the secretion of steroid hormones. After a 7- day treatment with ACTH the *in vitro* cells appear to have an ultrastructure completely similar to that of the corresponding zones of the adrenal cortex *in vivo*.

Example VII

Culture of normal human adult tenocytes (tendon fibroblasts)

The aim of the procedure is to isolate the tenocytes or fibroblasts resident in the tendons – structures consisting of densely packed highly ordered collagen fibres with quite few interspersed blood vessels.

The tendon sample taken intra-operatively with the informed consent of the patient is maintained at 4°C until it reached the laboratory. It is then immediately transferred to a Petri dish and chopped thanks to the scissor action of two very sharp knives. The fragments are subjected to enzymatic digestion in a solution of trypsin (0.25% w/v), type I collagenase (3% w/v) and ovine testicular hyaluronidase (0.25% w/v) in an incubator at 37°C with the help of a magnetic stirrer maintained at low speed (50 rpm) for 30 minutes. The fraction is then centrifuged at 600 rpm for 10 minutes at 4°C. The supernatant is decanted, the pellet is resuspended and the living cells are counted. The tenocytes are then seeded.

Supports used for the* in vitro *cultures

1. Plastic flasks with treated or untreated surfaces to increase cell adhesiveness

2. Fibroin membranes (pure or combined with other compounds)

Culture medium

The culture medium used is Dulbecco's Modified Eagle Medium (DMEM) enriched with inactivated FBS (20% v/v), cephaloridin (50 μg/ml), streptomycin (50 μg/ml) and nystatin (25 UI/ml). The medium can also be enriched with *platelet derived growth factor* (PDGF) and *fibroblast growth factor* (FGF).

Evolution of the cultures

The normal human tenocytes proliferate rapidly, first forming a continuous single layer of cells that are arranged parallel to each other and then a series of layers with parallel cells that form fairly long vortices. In the meantime, the tenocytes produce and secrete onto the supports considerable amounts of type I collagen fibre precursors, which are then integrated into relatively large fibre bundles. The cytoplasm contains type I collagen fibre precursors, decorin and intermediate filaments made up of vimentin. Like other fibroblasts, the tenocytes are avid consumers of the glucose present in the culture medium and, typically, secrete considerable amounts of lactic acid into the medium.

Example VIII

Culture of normal human adult chondrocytes

Fragments of articular cartilage are obtained from patients who have given their informed consent. The tissue is maintained at 4°C until it reaches the laboratory. It is then transferred to a Petri dish and cut to fragments measuring 1-3 mm^3 in volume by two very sharp knives. The chondrocytes (or cartilaginous cells) are contained in a highly viscous extracellular matrix. The cartilaginous fragments are therefore incubated in 5 ml of a solution of ovine testicular hyaluronidase (0.5% w/v) in Hanks' Basal Salt Solution (BSS) for 15-20 minutes at room temperature (18°C). The fragments are then washed in BSS and transferred to an Erlenmeyer flask containing 5 ml of a trypsin solution (0.2% w/v) which is slowly stirred for 30 minutes. The supernatant is decanted and a 5 ml solution of collagenase (0.25% w/v) in BSS is next added. After a 30-min incubation at 37°C, the supernatant is decanted, a further 5 ml of collagenase solution (0.25% w/v) in BSS is added and the fragments are incubated for 120 minutes at 37°C. The pre-digested fragments are triturated with Pasteur pipettes having gradually decreasing bores. The supernatant is removed and centrifuged at 600 g for 9 minutes. The sedimented cells are centrifuged again after their re-suspension in BSS. The isolated cells are then re-suspended, counted and seeded onto the culture supports.

Supports used for the* in vitro *cultures

1. Plastic flasks with treated or untreated surfaces to increase cell adhesiveness

2. Fibroin membranes (pure or combined with other compounds)

Culture medium

The culture medium used is Ham's F12 with the addition of FBS (15% v/v), penicillin (100 U/ml), streptomycin (100 U/ml) and Mg^{2+} (2.3 mM).

Evolution of the cultures

The human chondrocytes cultured on two-dimensional substrates appear as spindle-shaped cells that proliferate rapidly, forming confluent monolayers of cells that are mainly arranged in parallel to one another. On the same substrates the production of the extracellular matrix components and of type II collagen is extremely limited. Only the culture on three-dimensional supports allows the cells, as their proliferating activity gradually attenuates, to differentiate and produce considerable quantities of histo-typical materials (glycosaminoglycans, etc.) proper of the extracellular matrix and of type II collagen fibres.

Example IX

Culture of normal human adult pre-adipocytes

The sample of white adipose tissue, taken intra-operatively after obtaining an informed consent from the patient, is repeatedly washed with *phosphate buffered saline* solution A (PBS/A) to remove most of the blood cells it contains, and then it is cut into pieces weighing around 10 mg each. The tissue pieces are subjected to a first round of enzymatic digestion with a disaggregating solution consisting of *phosphate buffered saline* solution A (PBS/A, 10mM), type I collagenase (1.5 mg/ml) and bovine serum albumin (BSA, 20 mg/ml) for 30-40 minutes at 37°C, under continuous stirring. The ratio between adipose tissue weight and disaggregating solution volume is 1g/4 ml. The pre-digested pieces of adipose tissue are then filtered through a nylon mesh with 250 µm diameter pores, subjected to a second round of enzymatic digestion with the disaggregating solution for a further 30 minutes at 37°C and filtered again through a nylon mesh as described above. The two cellular suspensions are then mixed and centrifuged at 200 g for 10 minutes. To eliminate the erythrocytes, which at this point constitute the main contaminating cell type, the cellular suspension is treated with an *erythrocyte lysing buffer* (ELB: NH_4Cl 154 mM, $KHCO_3$ 10 mM, EDTA 0.1 mM) at room temperature (18-20°C) for 10 minutes, and subsequently filtered through a nylon

mesh with 150 µm diameter pores. After repeated washings and centrifugations (at 200 g for 10 minutes), the isolated cells are resuspended, counted and seeded onto the culture supports.

Supports used for the* in vitro *cultures

1. Plastic flasks with treated or untreated surfaces to increase cell adhesiveness

2. Fibroin membranes (pure or combined with other compounds)

Culture medium

The culture medium used for the human pre-adipocytes is a defined ratio combination of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 (1:1 v/v), containing penicillin (100 U/ml), streptomycin (0.1 mg/ml) and FBS (10% v/v).

Evolution of the cultures

To remove the cellular debris, the cultures are washed with PBS/A after a 16-20 hour incubation period. Fresh culture medium for pre-adipocytes is now added (DMEM/ Ham's F12 1:1 v/v enriched with $NaHCO_3$ 15 mM, HEPES 15 mM, biotin 33 µM, pantothenic acid 17 µM, iron-saturated human transferrin 10 µg/ml, penicillin 100 U/ml and streptomycin 0.1 mg/ml).

The pre-adipocytes have a fibroblast-like morphology but they emit numerous, often velamentous, cytoplasmic expansions. They proliferate, at first slowly and then more rapidly, but in the conditions described so far they do not differentiate. To induce and maintain adipose differentiation, which implies an irreversible block of the proliferative activity and the progressive accumulation of lipids in the cytoplasm until a signet ring appearance is achieved, typical of the mature adipocytes, the pre-adipocyte cultures are treated with the following compounds added to the growth medium: cortisol (100 nM), insulin (66 nM), triiodothyronine (0.2 nM) and, for the first three days, with isobutylmethylxanthine (IBMX, 0.25 mM).

Example X

Culture of human adult smooth muscle cells

The smooth muscle cells can be isolated from the *tunica muscularis* of hollow viscera (e.g. the intestine) or from blood vessels (e.g. arteries of various diameter and site and veins from the lower half of the body of adult subjects). The visceral or vascular *tunicae*

musculares are isolated from intra-operative samples (after obtaining an informed consent from the donors), placed in Dewar flasks at 4°C and sent to the laboratory. The samples are then repeatedly washed with *phosphate buffered saline* solution A (PBS/A) to remove the blood, cut into pieces of 1-2 mm^3 volume and, finally, subjected to repeated rounds of enzymatic digestion. The dissociating solution consists of Hanks' Basal Salt Solution (BSS) containing trypsin (0.25% w/v) and collagenase (5% w/v). Dissociation is carried out at room temperature (18°C) with a slow but constant stirring (60 rpm). The supernatants are enriched with anti-protease agents and FBS (20% v/v), stored at 4°C, mixed together and finally centrifuged at 100 g for 4 minutes. The pellets are washed in BSS and centrifuged again several times. The isolated cells are then resuspended, counted and inoculated on the culture supports.

Supports used for the* in vitro *cultures

1. Plastic flasks with treated or untreated surfaces to increase cell adhesiveness

2. Fibroin membranes (pure or combined with other compounds)

Culture medium

The culture medium used is Dulbecco's Modified Eagle Medium (DMEM) with the addition of penicillin (100 U/ml), streptomycin (0.1 mg/ml) and FBS (10% v/v).

Evolution of the cultures

The smooth muscle cells have a spindle-shaped appearance, they have a single nucleus and proliferate actively *in vitro* forming confluent monolayers of cells. They express type-specific isoforms of actin. They can be easily sub-cultured after detachment with a trypsin solution (0.25% w/v) in Hanks' BSS.

Example XI

Culture of normal endothelial cells of blood vessels

The endothelial cells form single layer lamina of squamous epithelial cells that cover the entire surface of blood vessels of various diameters. The human endothelial cells are generally isolated from intra-operative samples (after obtaining informed consent) of umbilical vessels, dermal tissue, adipose tissue, *etc*. Here, we will limit ourselves to describing the method for isolating them from the umbilical vessels.

Ten cm sections of these aseptically removed vessels are placed in Dewar flasks with

melting ice and taken to the laboratory. One end of the vessel is fixed to a syringe which first injects *phosphate buffered saline* solution A (PBS/A) into the vessel. The umbilical vessel is then filled with dissociating solution (containing collagenase 0.25% w/v in Hanks' Basal Salt Solution [BSS]), the free end is clamped and it is left to incubate for 20 minutes at room temperature (18°C). The clamp is removed, the dissociating solution is drained off and poured, together with the PBS/A used to wash out the vessel, into a conical test tube and centrifuged for 5 minutes at 4°C at 100 *g*. The isolated cells are resuspended in BSS and centrifuged twice. Finally, the spun down cells are counted and applied to the culture supports.

Supports used for the* in vitro *cultures

1. Plastic flasks with treated or untreated surfaces to increase cell adhesiveness

2. Fibroin membranes (pure or combined with other compounds)

Culture medium

The culture medium generally used for endothelial cells is Dulbecco's Modified Eagle Medium (DMEM) with the addition of penicillin (100 U/ml), streptomycin (0.1 mg/ml) and FBS (10% v/v). It is also possible to use Medium 199 or medium RPMI, with the same additions. The human endothelial cells also require the presence of hypothalamus-derived endothelial mitogens (25 µg/ml) and heparin (90 µg/ml) in the culture medium.

Evolution of the cultures

The human endothelial cells in culture have a triangular or polygonal shape with raised margins which confers them their typical halo due to the associated light diffraction. They proliferate very intensely if human serum (up to 20% v/v) is added to the medium together with the several other components mentioned above, instead of bovine serum. The cells form a continuous monolayer that eventually covers the entire surface of the culture support. The cells can now be sub-cultured by means of a standard trypsinization (trypsin 0.25% w/v in BSS). The human endothelial cells typically produce factor VIII and express specific surface antigens, all detectable with immunocytochemical techniques. They also express the converting enzyme, angiotensin, and uptake the low density lipoproteins, as can easily be detected with biochemical methods. Proliferation of the endothelial cells stops spontaneously if they

are allowed to reach confluence (an example of contact inhibition of growth).

The invention described above refers to some of its particular forms of embodiment.

It is however evident that the limitation is not limited to these forms of embodiment, but includes all the modifications and variations that can be considered and which do not require the application of any inventive effort, thus without going beyond the framework of this invention as claimed.

In accordance with other preferred forms of realisation, the substrate consists of a mixture of bio-resorbable polymers in which fibroin is present in a quantity varying from 0% to 100% in weight.

CLAIMS

1. Substrate suitable to be used as an artificial transplanted tissue able to achieve a complete and optimum integration with the other cell systems and their functions of the organism in which the transplant or the implant has been made, and able to accomplish for the survival, the proliferation and the correct differentiation of specialised tissue cells of the human body, said substrate consisting of a material that is bio-compatible and bio-resorbable in pre-determinable times, in order, or with which a functional connection has been established, said substrate comprising a mixture and/or combination of natural and/or synthetic polymers, wherein this mixture and/or combination includes fibroin.

2. Substrate according to claim 1, wherein said fibroin is of natural origin.

3. Substrate according to claim 2, wherein said fibroin is secreted by the *Bombyx mori* silkworm.

4. Substrate according to claim 1, wherein said fibroin is of synthetic origin.

5. Substrate according to anyone of the preceding claims, wherein said mixture contains pure fibroin.

6. Substrate according to anyone of claims 1 to 4, wherein said mixture contains fibroin derivatives.

7. Substrate according to anyone of the preceding claims, wherein the fibroin covers a supporting scaffold advantageously and uniformly, said supporting scaffold consisting of a ceramic, a metal and/or a synthetic and/or natural polymeric material and by any of their combinations.

8. Substrate according to claim 7, wherein the fibroin is deposited on said supporting scaffold.

9. Substrate according to claim 7, wherein the surface of said supporting scaffold is covered with fibroin.

10. Substrate according to anyone of the preceding claims, wherin the fibroin is present in said substrate in a quantity varying from 20% to 80% in weight.

11. Substrate suitable to be used as an artificial transplanted tissue able to achieve a complete and optimum integration with the other cell systems and their functions of the organism in which the transplant or the implant has been made, able to accomplish for the survival, the proliferation and the correct differentiation of specialised tissue cells of the human body, said substrate consisting of a material that is bio-compatible and bio-resorbable in pre-determinable times, in order, or with which a functional connection has been established, whereby said substrate consists of pure fibroin.

12. Substrate according to anyone of the preceding claims, wherein human body tissue cells are seeded on it with the aim of favouring their growth, said tissue cells including those of the skin and of the liver, mesothelial cells, astrocytes, human skeleton osteoblasts, tenocytes or human tendon fibroblasts, chondrocytes or cells isolated from cartilaginous tissues, endothelial cells of blood vessels, steroid-secreting cells of the adrenal cortex, smooth muscle cells of the *tunica muscularis* of the intestine and blood vessels, squamous epithelial cells of the oral cavity or the conjunctiva/cornea and human pre-adipocytes of the white adipose connective tissue.

13. Use of fibroin for the production of a substrate according to anyone of the preceding claims, said substrate being suitable for the survival, the proliferation and the correct differentiation and functioning of specialised tissue cells of the human body both inside and outside of it.

14. Use of fibroin according to claim 12, wherein the human body tissue cells include those of the skin and of the liver, mesothelial cells, astrocytes, human skeleton osteoblasts, tenocytes or human tendon fibroblasts, chondrocytes or cells isolated from cartilaginous tissues, endothelial cells of blood vessels, steroid-secreting cells of the adrenal cortex, smooth muscle cells of the *tunica muscularis* of the intestine and blood vessels, squamous epithelial cells of the oral cavity or the conjunctiva/cornea and human pre-adipocytes of the white adipose connective tissue.

15. Process for the production of a substrate according to anyone of the preceding claims, wherein degummed silk is dissolved in a solution of lithium bromide in water at a temperature higher than room temperature and at a standard pressure, the solution so obtained being then filtered through a porous ceramic filter, diluted with distilled water, dialysed and left to evaporate in polystyrene containers, wherein the membrane so obtained is immersed in a solution of methanol and water to make it crystalline and hence insoluble in water.

BIO-ARTIFICIAL SUBSTRATE FOR THE PRODUCTION OF ANIMAL AND, IN PARTICULAR, HUMAN TISSUES AND ORGANS

Abstract

A substrate suitable for the survival, the proliferation and the correct differentiation and functioning of specialised tissue cells of the human and animal body, consisting of a material that is bio-compatible and bio-resorbable in pre-determinable times, which can be transplanted or implanted onto or connected with the body in order to achieve a complete integration of the transplanted, implanted or connected tissue with the other cell systems and their functions in the organism onto which the transplant or the implant or with which the connection has been made, and comprising a mixture and/or combination of natural and/or synthetic polymers in which fibroin is present.

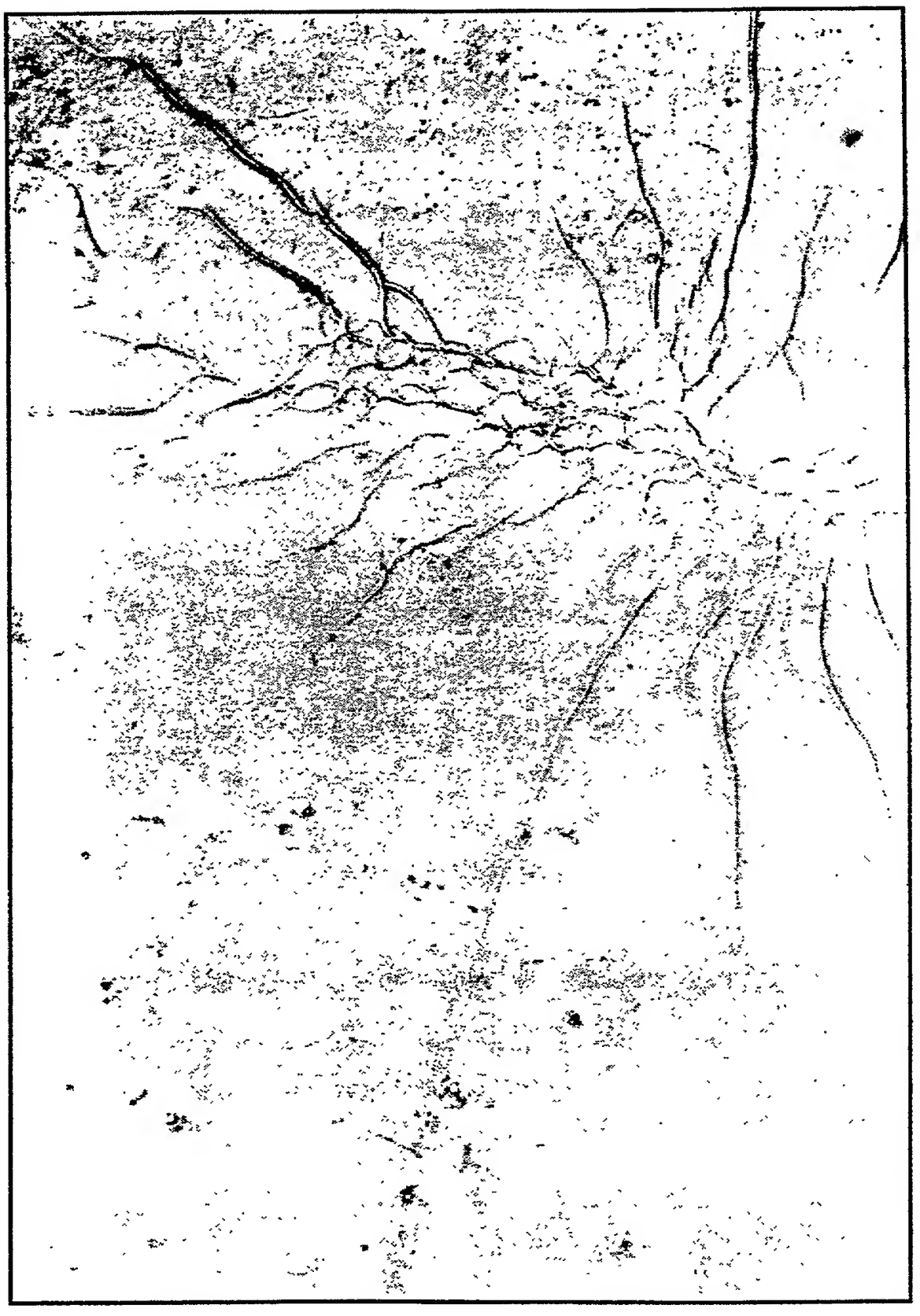


Fig. 1

Fig. 2

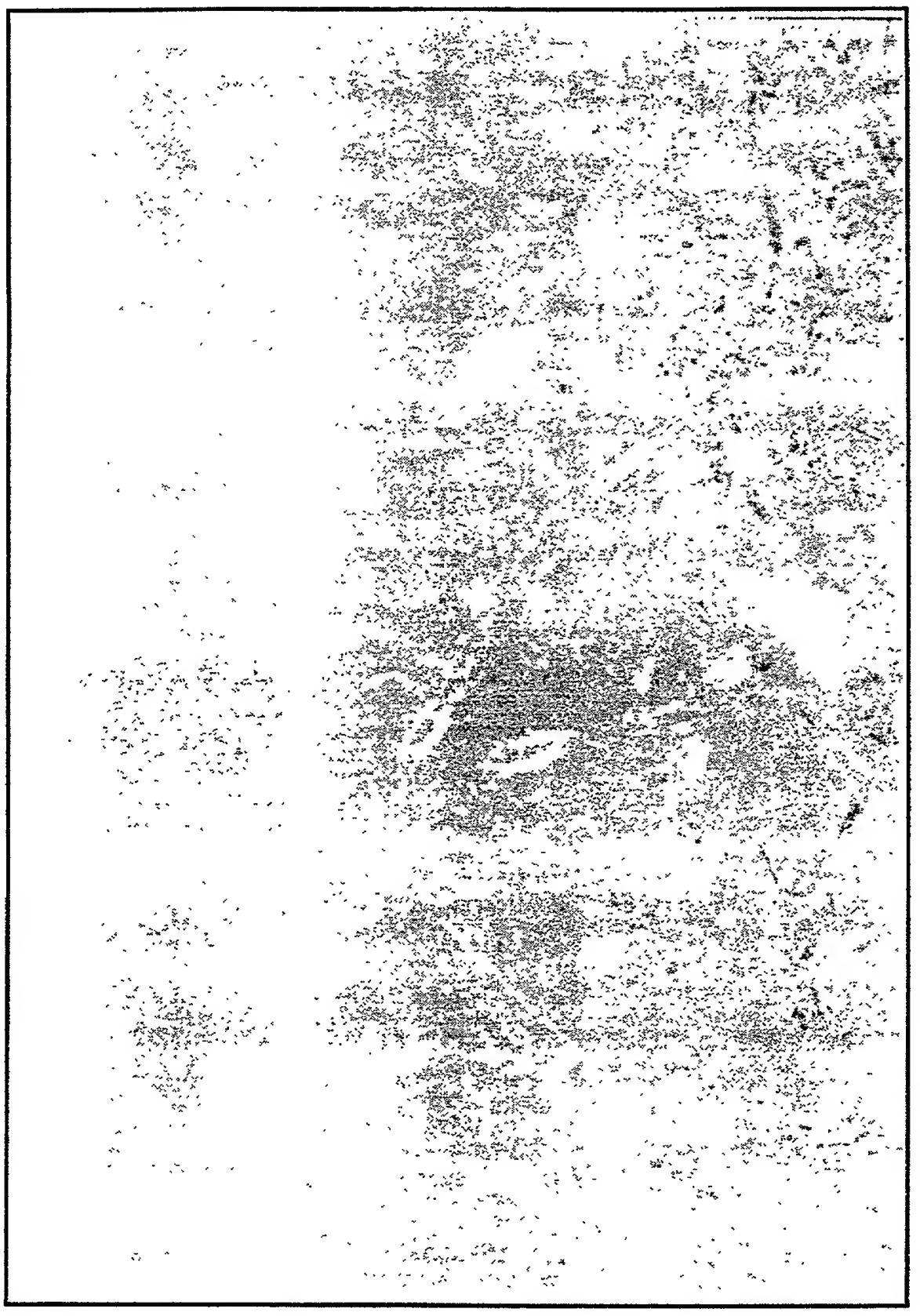


Fig. 3

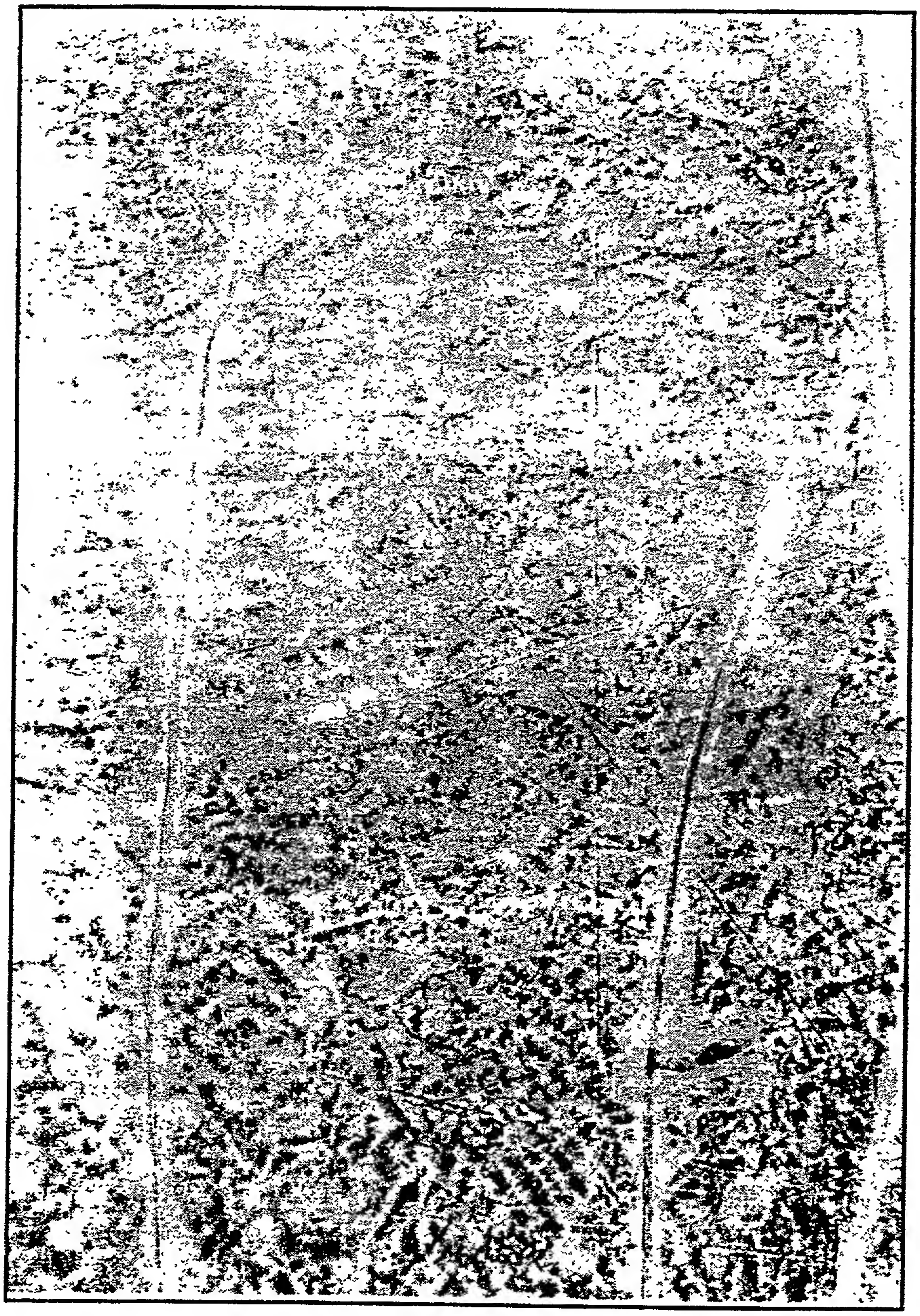


Fig. 4

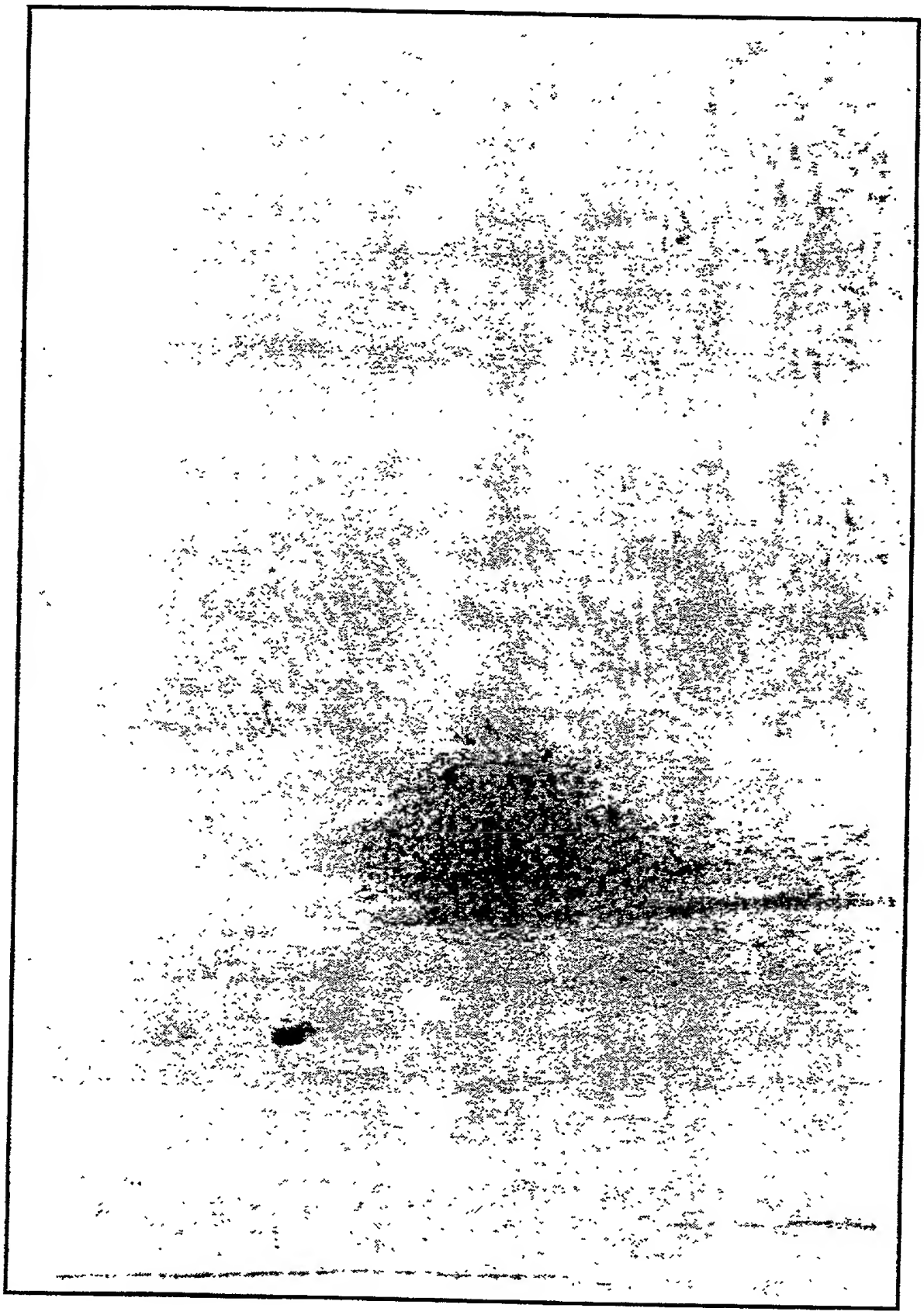
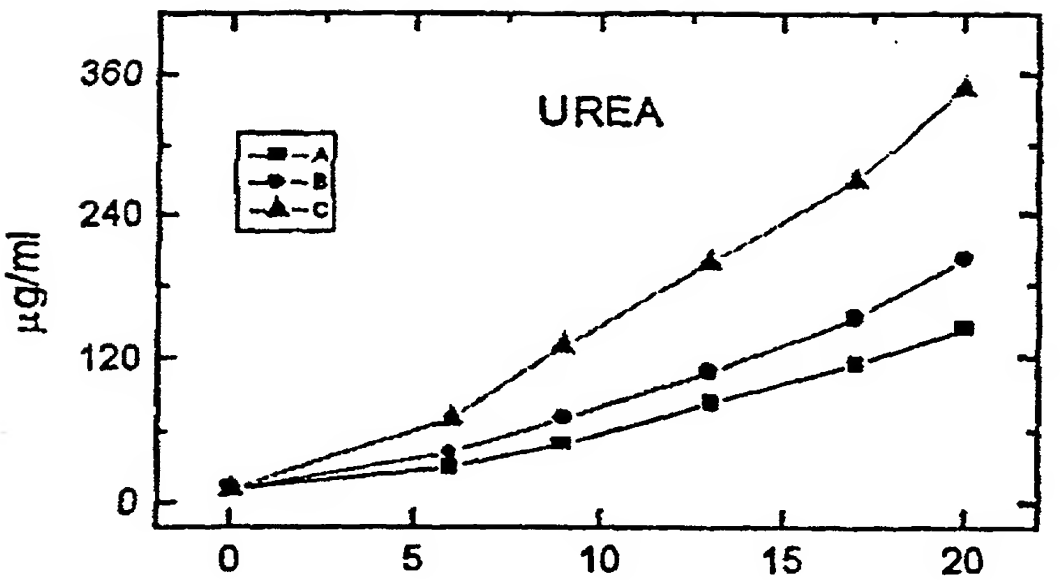


Fig. 5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B
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120
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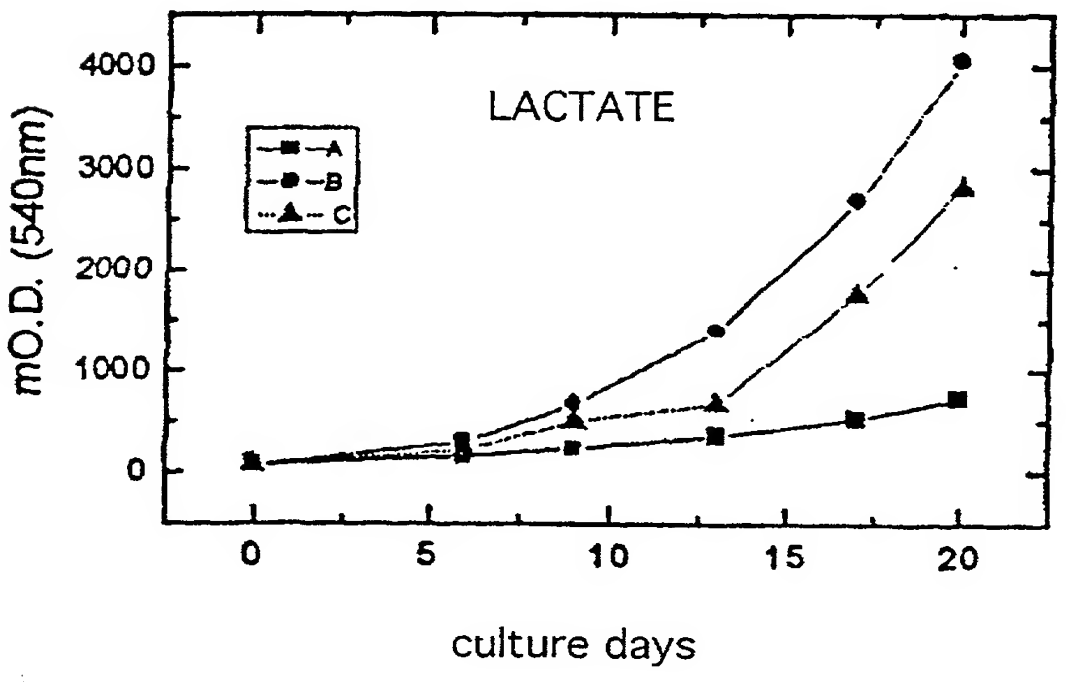
µg/ml
0
5
10
15
20

Fig. 6


LACTATE
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B
C
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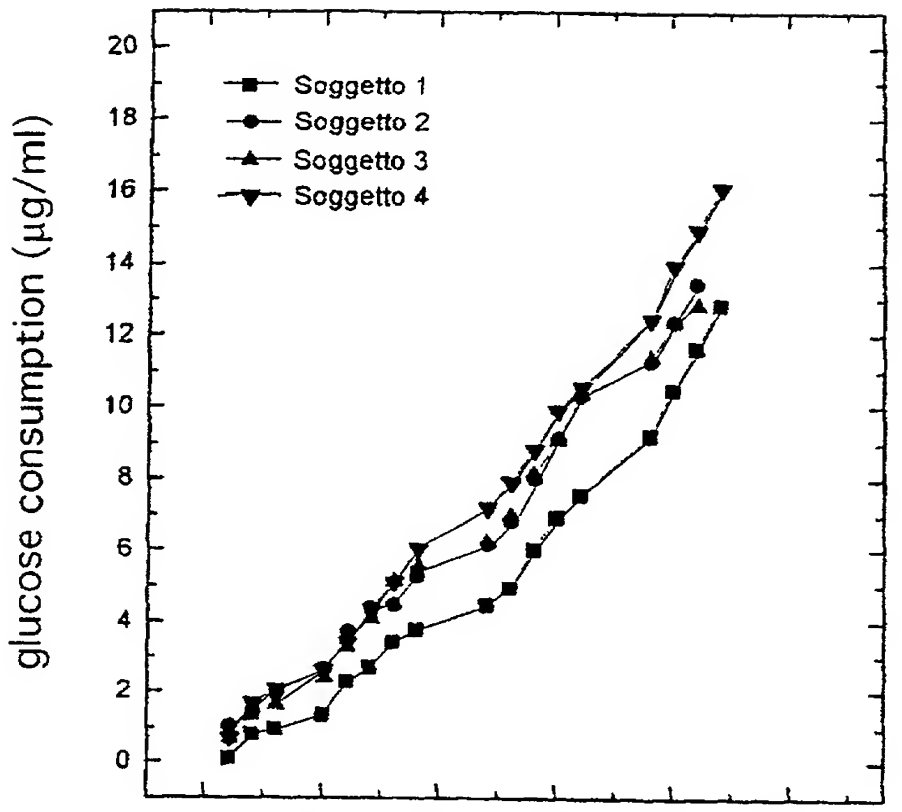
3000
2000
1000
0
mO.D. (540nm)
0
5
10
15
20
culture days

Fig. 7


glucose consumption (µg/ml)
culture days
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Soggetto 2
Soggetto 3
Soggetto 4
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4
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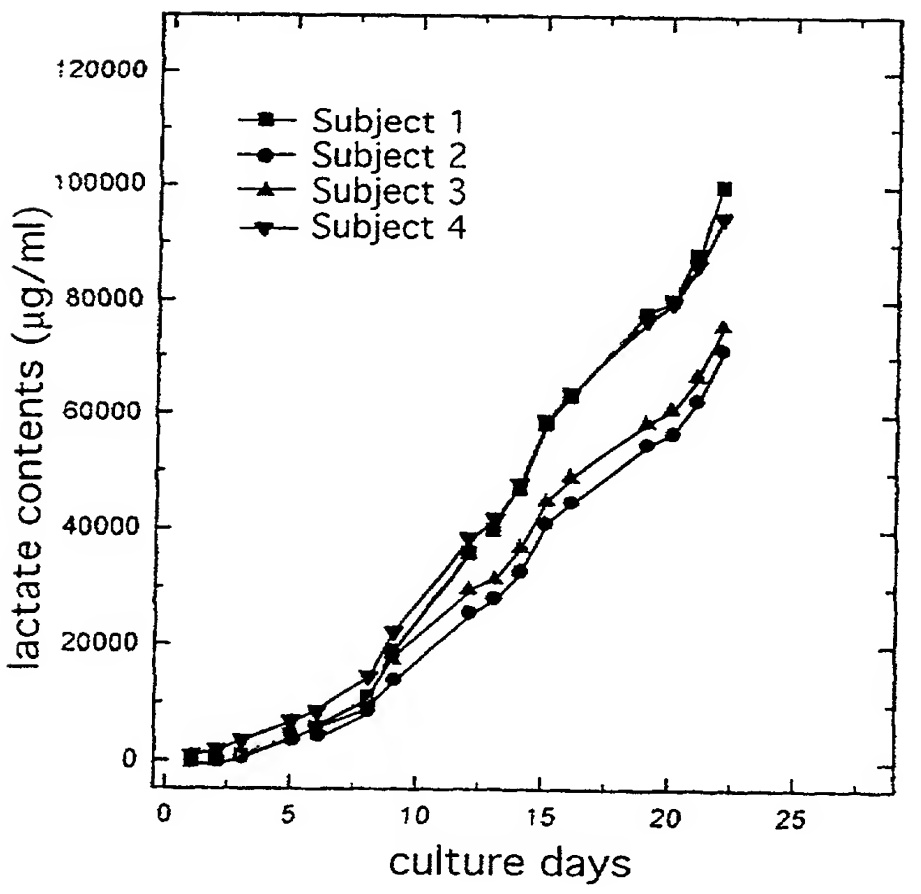
12
14
16
18
20
0
5
10
15
20
25

Fig. 8


lactate contents (µg/ml)
culture days
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Subject 2
Subject 3
Subject 4
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40000
60000
80000
100000
120000
0
5
10
15
20
25

Fig. 9

Fig. 10

Fig. 11

Fig. 12

Fig. 13

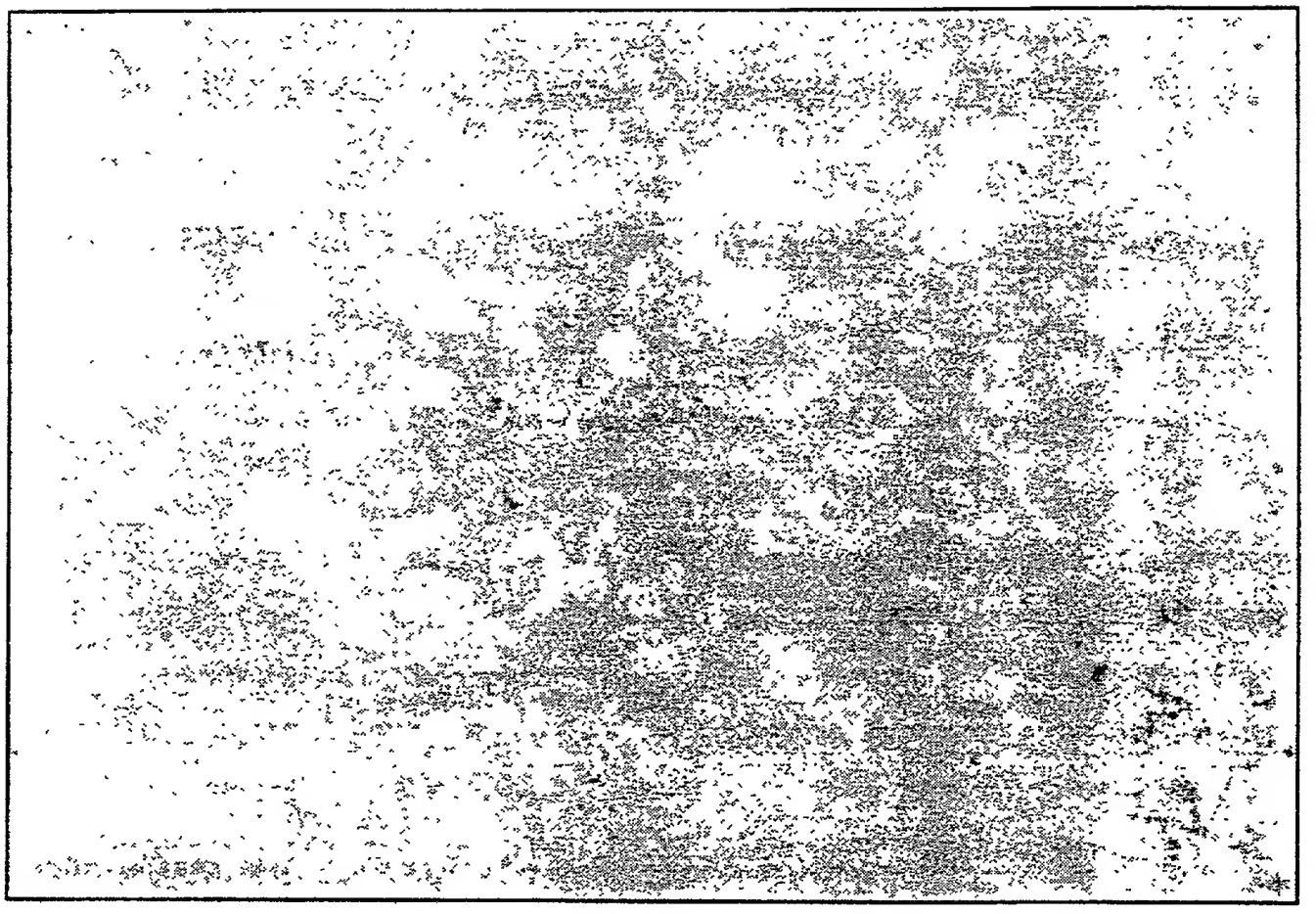

Fig. 14

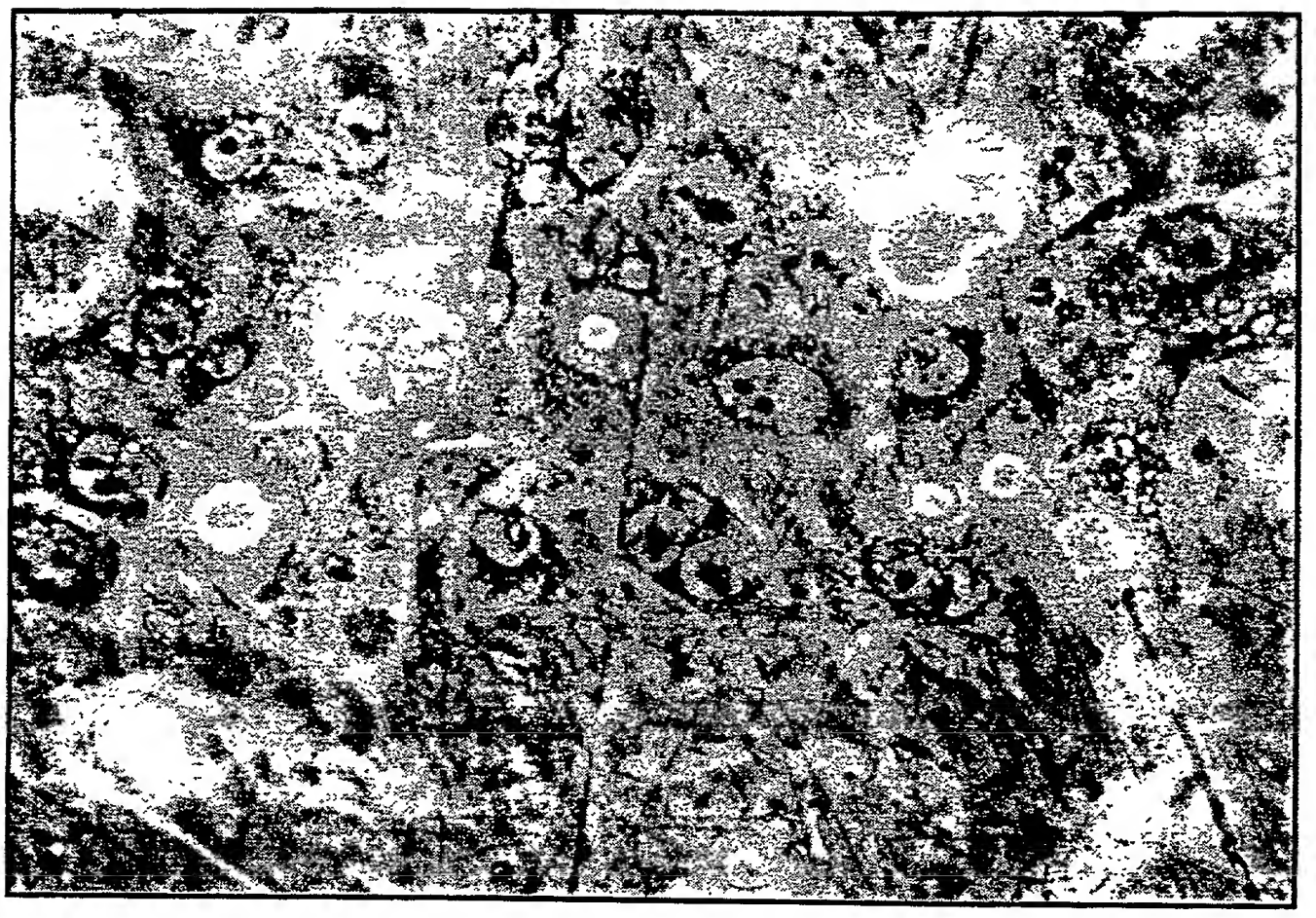

Fig. 15

Fig. 16

Fig. 17

Fig. 18

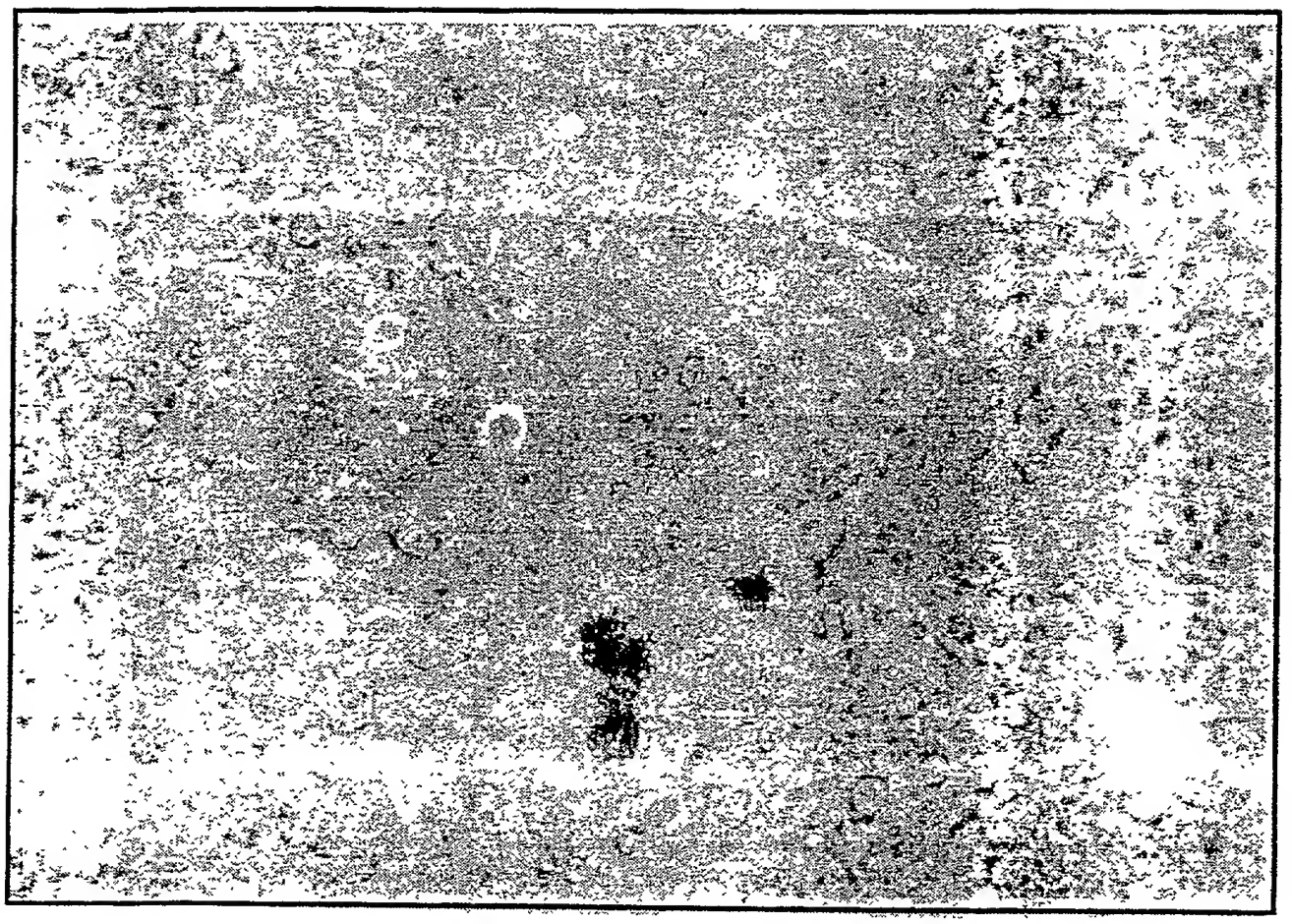

Fig. 19

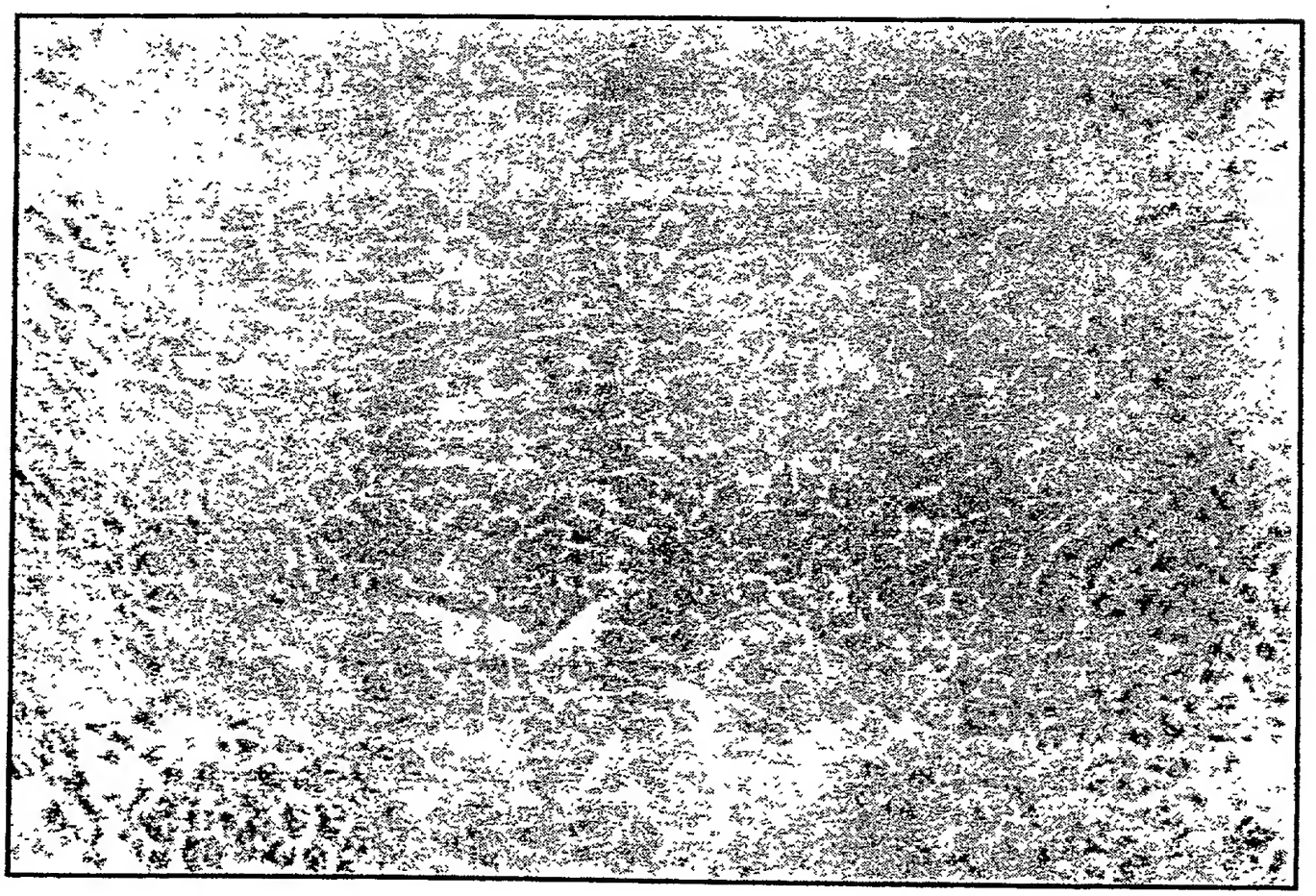

Fig. 20

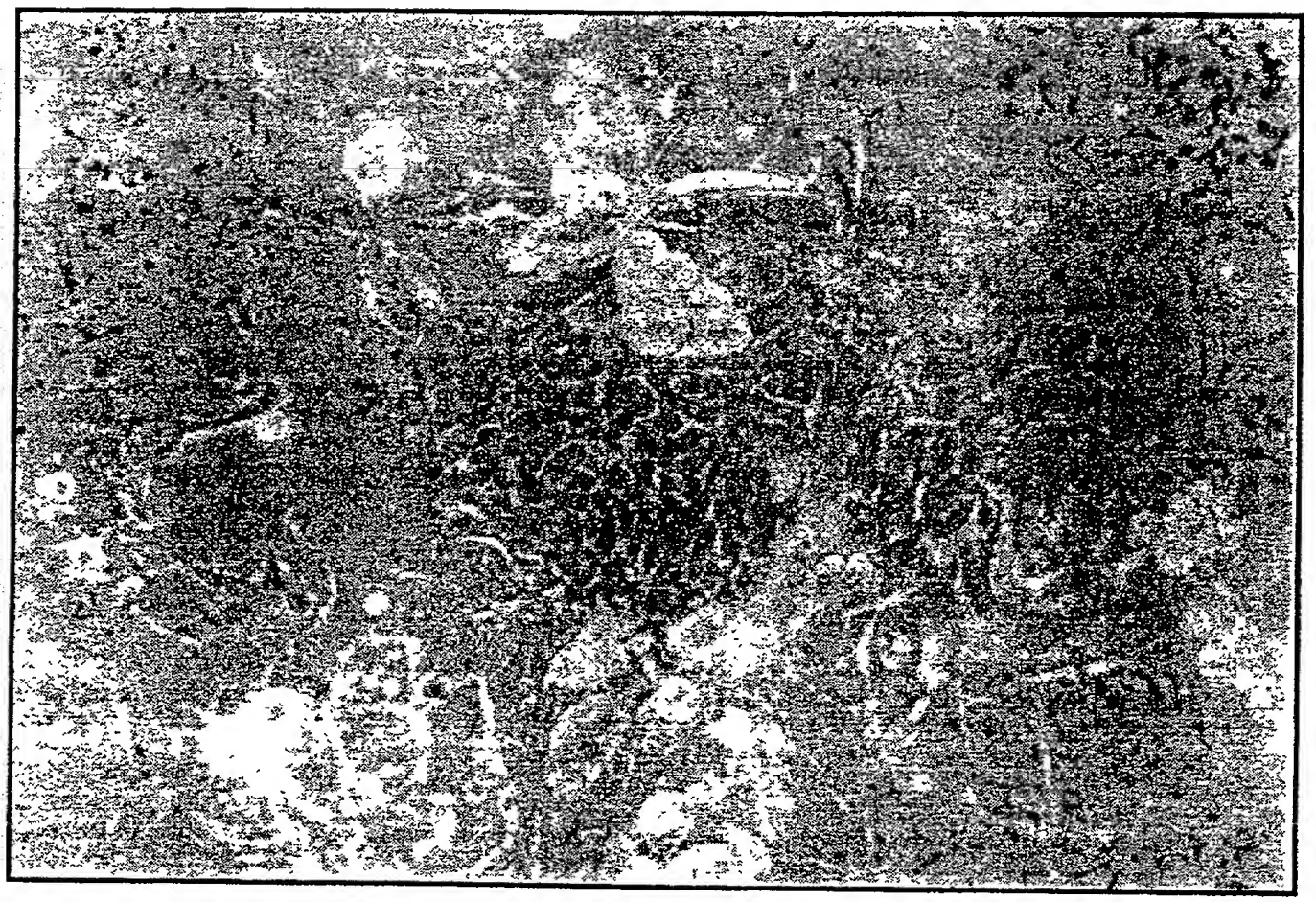

Fig. 21

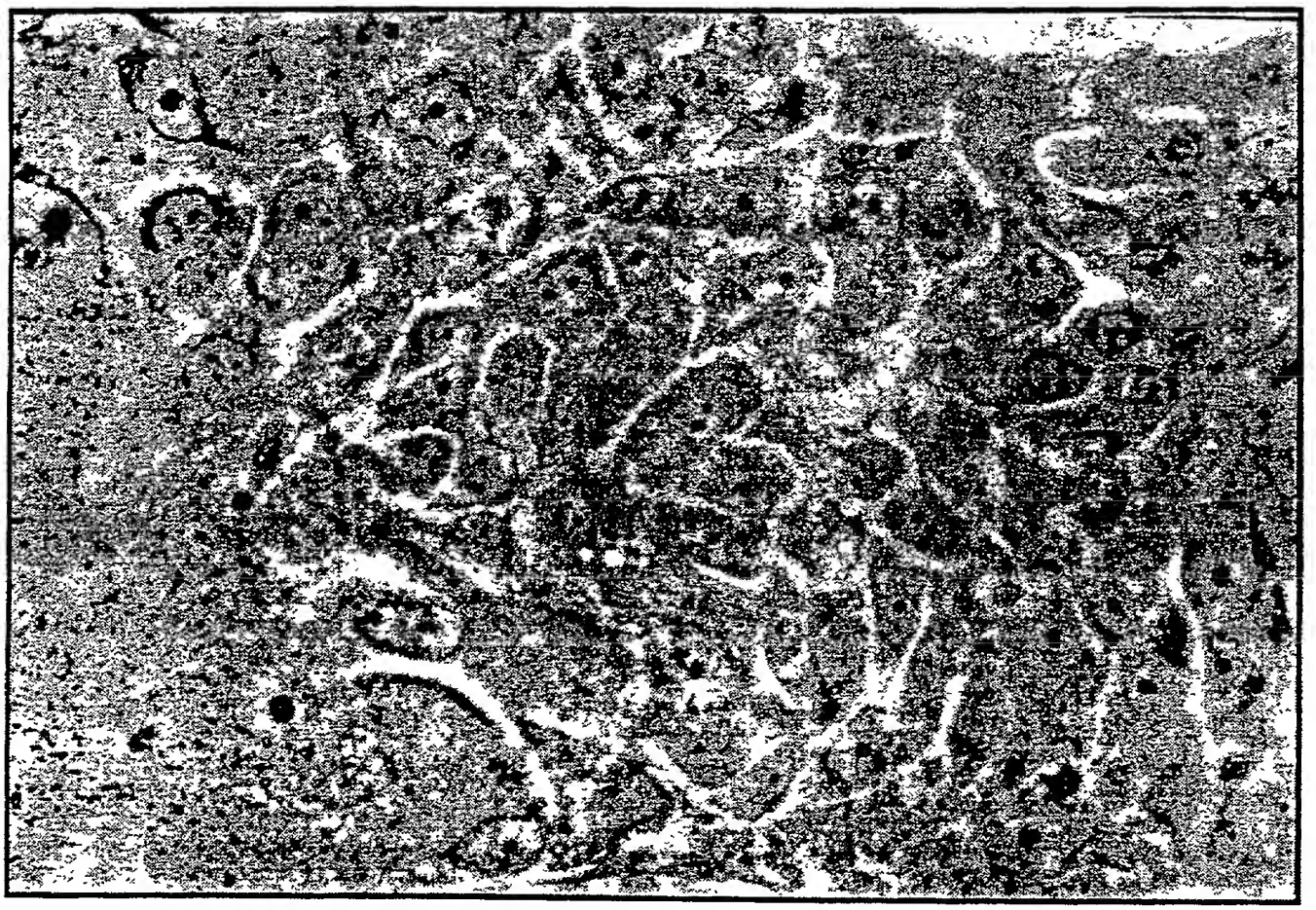

Fig. 22

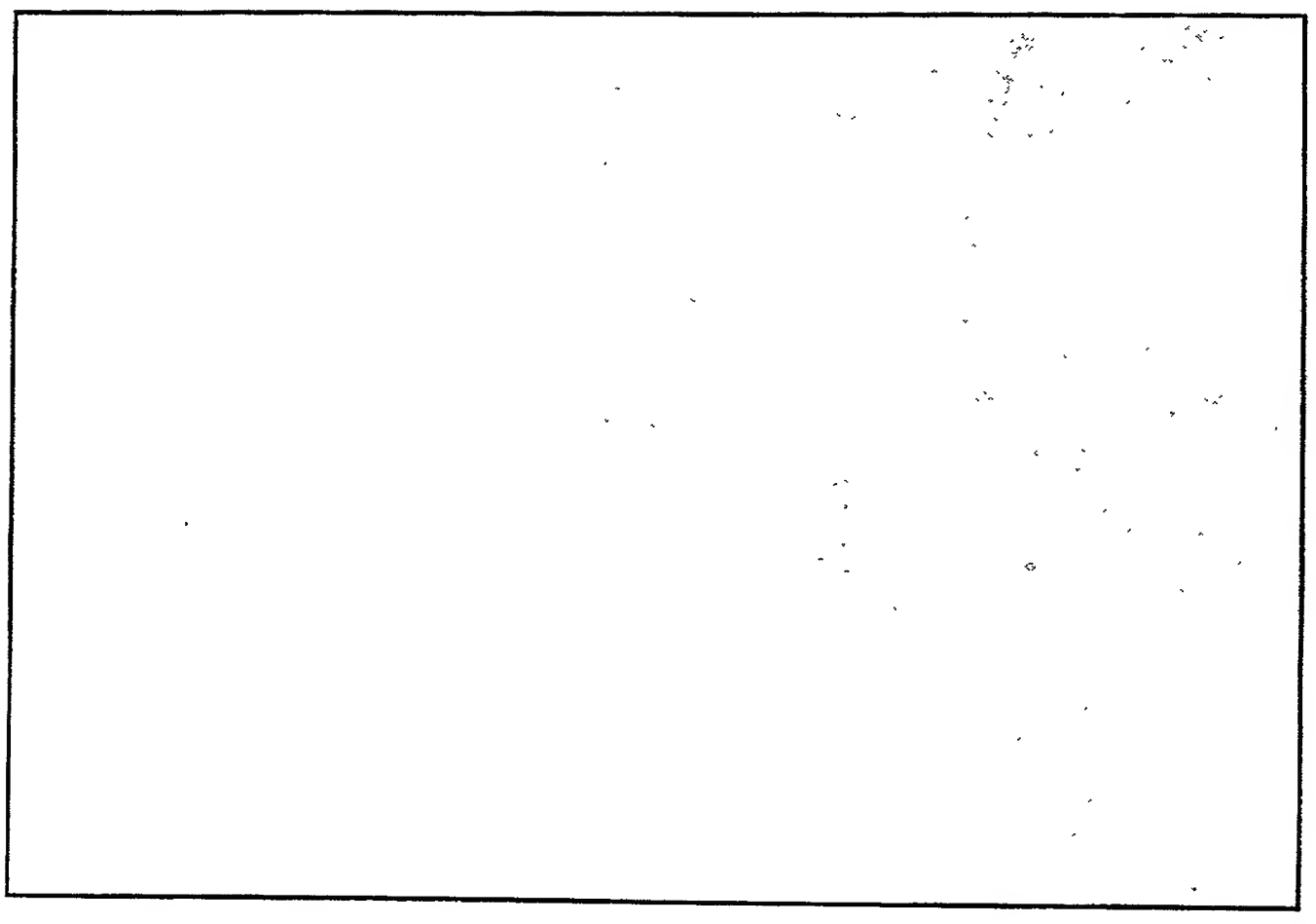

Fig. 23

Fig. 24

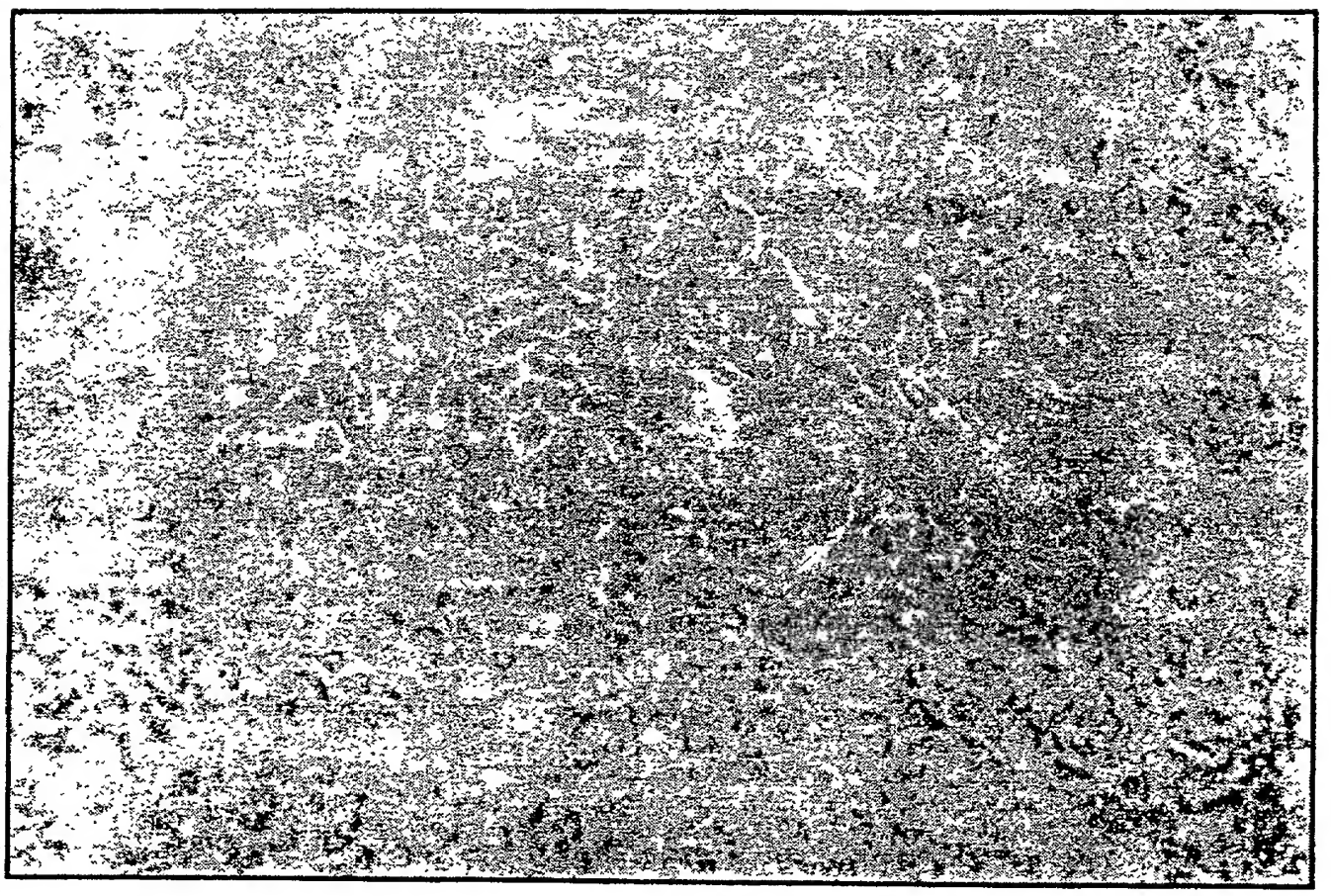

Fig. 25

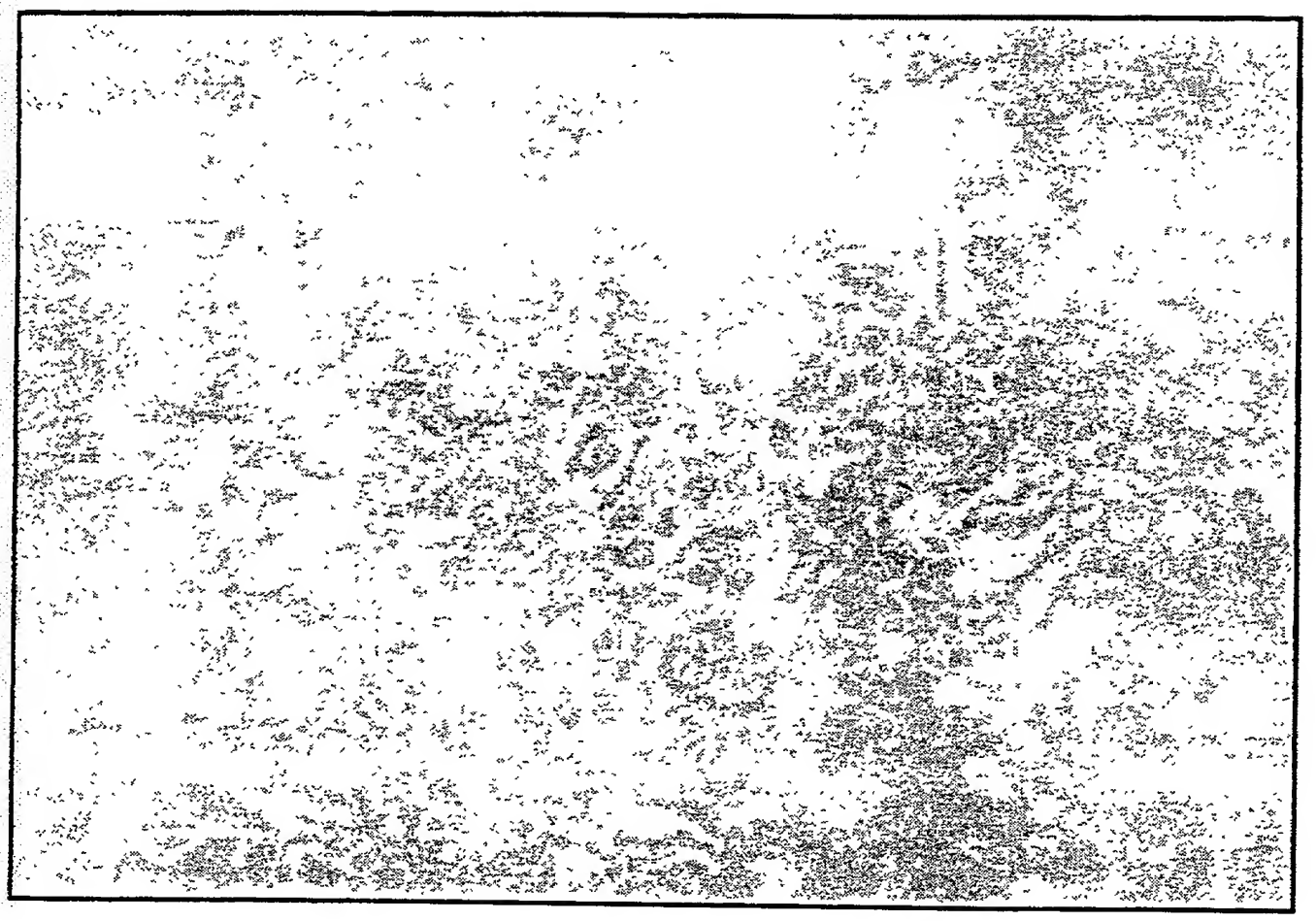

Fig. 26

Docket No. 17642-59

DECLARATION, POWER OF ATTORNEY AND PETITION (WITH AUTHORIZATION RE AGENT)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **BIO-ARTIFICIAL SUBSTRATE FOR THE PRODUCTION OF ANIMAL AND, IN PARTICULAR, HUMAN TISSUES AND ORGANS** the specification of which was filed on 28.September.2000 as Application Serial No. PCT/IT00/00382 and was amended on 27 December 2001.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 C.F.R. § 1.56(a).

I hereby claim foreign priority benefits under 35 U.S.C. § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application of which priority is claimed.

PRIOR FOREIGN APPLICATION(S)

Number	Country	Filing Date	Priority Claimed?
VR99A000082	IT	01.October.1999	Yes

I hereby declare: All statements of my own knowledge are true, and I believe that all statements made on information and belief are true. I make these statements with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued on the application.

POWER OF ATTORNEY

I appoint the following as my attorneys with full power of substitution and revocation, to prosecute said application and to transact in connection therewith all business in the Patent and Trademark Office and before competent International Authorities:

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Bosworth, M.K., Reg. 28,186
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McKinley, D., Reg. 42,867
McRoss, L., Reg. 40,427
Morton, C.A., Reg. 44,954
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Sherry, J., Reg. 43,918
Smith, G.P., Reg. 20,142
Swienton, B., Reg. 49,030
Valencia, R.A., Reg. 43,216
Wood, G.B., Reg. 28,133
Wrigley, B.A., Reg. 34,950

46

Docket No.

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I further authorize and empower my United States attorneys, OPPENHEIMER WOLFF & DONNELLY LLP, to receive and act upon all instructions provided to them by my the following agents , Europatent – Euromark srl with respect to all matters relating to the filing, prosecution, abandonment, or issuance of the above-described U.S. patent application including all divisional, continuing, or other related applications. OPPENHEIMER WOLFF & DONNELLY LLP may continue to follow all instructions received from the agents until notified in writing to the contrary.

Wherefore I pray that Letters Patent be granted to me for the invention or discovery described and claimed in the foregoing specification and claims, and I hereby subscribe my name to the foregoing specification and claims, declaration, power of attorney, and this petition.

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Inventor's signature _____ Date 28 March 2002
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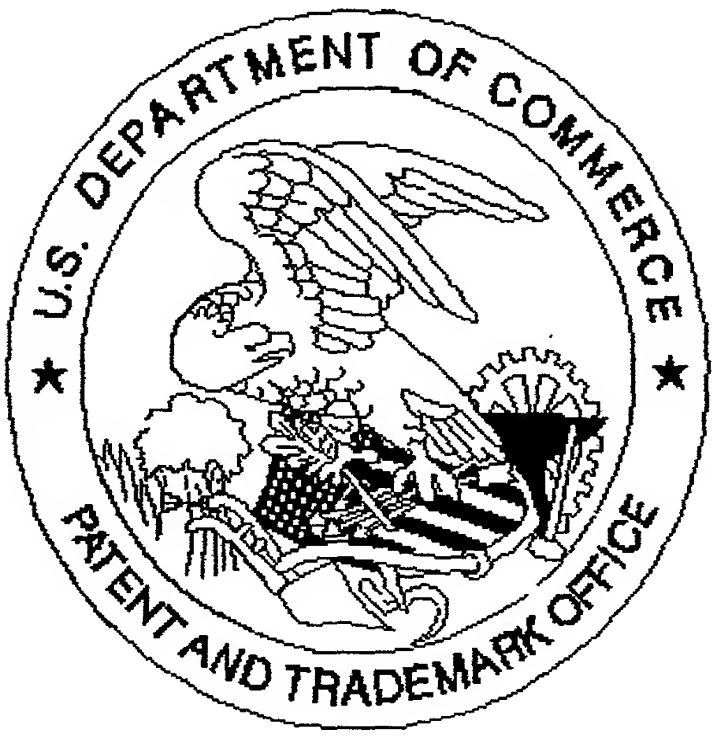
United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division



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